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Effect of N-methylethylphenylbarbituric Acid (Prominal) on the Cholesterol Content in the Ovarian Interstitial Gland of the Rat

By

LENNART CLAESSON

In his studies on the effect of Prominal on the ovary WESTMAN (1943, 1947) has suggested that this barbiturate has a depressant action on the gonadotrophic functions of the pituitary-hypothalamic system. This suggestion was born out by a series of investigations by the Durham school (EVERETT and SAWYER, 1949, 1950, SAWYER, MARKEE and EVERETT, 1950, MARKEE, EVERETT and SAWYER, 1952, EVERETT, 1952). It is evident from these investigations that it is possible to block ovulation in the rat ovary day after day by treatment with barbiturates, and that this effect is caused by prevention of the cyclic release of pituitary gonadotrophins.

In a previous work on the cholesterol in the ovarian interstitial cells (CLAESSON and HILLARP, 1947 b), cholesterol in the rat ovary was shown to undergo quantitative, cyclic changes. An investigation of the cholesterol alterations in ovaries at prevention of the cyclic gonadotrophin secretion by barbiturate sedation would therefore seem to be of interest.

Material and Methods

Twenty adult female albino rats, inbred for eight generations were used in these experiments. Vaginal smears were prepared regulary every morning throughout the experimental period. Part of the vaginal content was removed from the midportion

of the vaginal cavity by means of a fine spatula. Great care was taken not to touch the cervix.

Twice daily the animals were injected subcutaneously with Prominal (N-methylethylphenylbarbituric acid) in form of its sodium salt in a 1.8 per cent aqueous solution. This barbiturate was chosen on account of its long-acting effect and comparatively low toxicity even at higher dosage, which makes it particularly suitable for experiments of long duration. The amount of Prominal injected was determined on each separate occasion on the basis of the general condition of the animal. The animals received as large a dose as possible in order to obtain a high degree of sedation; not larger, however, than to enable the animals to take care of their nutrition, excretion and so on. In a few instances of high somnolence and persistent hypnosis, the dose was adjusted downwards and the animals were given water, electrolytes and glucose by means of a stomach tube. Throughout the whole experimental period the animals were carefully attended to in order to avoid malnutrition, dehydration, hypothermia, retention of urine, intercurrent infections, etc.

Treatment with Prominal was initiated at different stages of the vaginal cycle, determined by smears taken from the animals during a period of time prior to the first day of injection. During the period of Prominal treatment, the vaginal smears were classified as positive if they showed cornified cells only. Occasional smears with only epithelial cells were denoted as positive if lying in a series of cornified smears. All other types of smears were classified as negative.

The effect of Prominal on the cholesterol content in the ovary was examined following 5 to 15 days' treatment. Pregnant mare serum gonadotrophin — PMSG (Antex Leo[1]) was administered to eleven of the rats 2 days before autopsy without interrupting the Prominal treatment. In these animals the right ovary was removed under ether anaesthesia immediately before the subcutaneous injection of 100 to 150 I.U. PMSG; the left ovary was removed 48 hours later.

The ovaries were fixed in 10 per cent formalin (kept over

[1] Mr. S. Wiström, A-B. Leo, Helsingborg, has kindly put this preparation at my disposal.

$CaCO_3$). Representative frozen sections (10μ) from all the ovaries were examined for cholesterol content by the Schultz technique. The remaining part of the ovaries were embedded in paraffin, sectioned (10μ) and stained in haematoxylin-eosin.

When applying the Schultz reaction, the oxidation was carried out by placing the frozen sections in 2.5 per cent ferric alum at +38° to 39° C. for 48 hours. With regard to the specificity of this recation, cf. CLAESSON and HILLARP (1947 a), EVERETT (1947) and CAIN (1950). At present there is no reason to suppose that the Schultz-positive substance found in the rat ovary is different from that stored in the rabbit ovary, where it has been shown to be identical with cholesterol (CLAESSON, DIZFALUSY, HILLARP and HÖGBERG, 1948). — In Tables I and II, the approximative cholesterol content is denoted by + to +++++. The maximal amounts observed are indicated by +++++.

Results

Thirteen of the twenty animals examined showed clearly altered vaginal cycle during the Prominal treatment. All the animals treated for 9 days or longer belong to this group. Concerning the vaginal cycle the remaining seven animals (No. 17—21, 26, 44 and 47) cannot be judged with certainty.

The disturbances in the vaginal cycle of the thirteen animals first mentioned were of two quite different types as is evident from Table I. During the 8—10 last days of treatment seven rats did not show any signs of cyclic changes in the vagina which constantly presented a dioestrous stage. On the other side a group of five animals (No. 2, 8, 9, 23 and 41) showed vaginal smears of mainly oestrous type.

At the time of autopsy the macroscopic appearence of uterus was registered in the nine animals (No. 2—20), not stimulated with PMSG. Animals No. 2, 8, and 9 presenting vaginal smears of the oestrous type, showed hyperaemia and fluid distension of the uterus. On the other hand the uterus in the remaining animals showed neither distension nor any signs of hyperaemia.

The ovaries of twelve animals, No. 2—26, were examined histologically with special regard to the interstitial gland cells.

TABLE I

Effect of Prominal (Sodium N-methylethylphenylbarbituric Acid, 1.8 per cent aqueous solution) on the Oestrous Cycle and on the Cholesterol Content in the Interstitial Gland of the Rat Ovary. Figures in Parentheses indicate the Number of Days with continously Negative and Positive Vaginal Smears respectively, counted from and including the last Day of the Experiment.

Animal No.	Vaginal Smear Stage at the 1st Day of Treatment	Daily Treatment			Vaginal Smears		Interstitial Gland
		Period Days	Dose Mg.		Days Negative	Days Positive	Schultz Reaction
			Average	Range			
2	Metoestrus	15	15.7	0–22.5	3	12(6)	++++
3	Metoestrus	10	12.3	0–22.5	8(6)	2	++++
6	Prooestrus	10	14.8	0–22.5	9(9)	1	++++
8	Dioestrus	14	16.9	0–31.5	5	9(4)	++++
9	Dioestrus	10	16.0	0–27.0	2	8(2)	++++
11	Oestrus	10	15.4	0–22.5	10(10)	0	++++
17	Prooestrus	6	23.0	18.0–31.5	5(5)	1	++++
18	Prooestrus	6	23.7	18.0–31.5	4(4)	2	++++
20	Oestrus	5	22.4	13.5–27.0	5(5)	0	++++
21	Oestrus	8	23.0	14.4–31.5	5(1)	3	++++
23	Dioestrus	8	21.1	18.0–27.0	2(1)	6	++++ (R) ++++ (L)
26	Prooestrus	8	22.6	18.0–27.0	5(1)	3	++++
41	Oestrus	5	24.6	18.0–31.5	0	5(5)	++++
42	Oestrus	11	27.8	18.0–36.0	9(8)	2	++++
44	Oestrus	5	23.1	9.0–27.0	3(3)	2	++++
45	Procestrus	9	26.0	18.0–31.5	8(8)	1	++++
46	Oestrus	10	23.6	18.0–27.0	9(8)	1	++++
47	Dioestrus	5	23.9	18.0–27.0	2(1)	3	++++
50	Oestrus	9	25.1	18.0–36.0	8(8)	1	++++
51	Oestrus	10	24.9	18.0–36.0	10(10)	0	++++

(In order to obtain representative frozen sections of the ovaries of rats No. 41—51, the whole ovaries were used up, and could not be examined in this way.) In nine of these twelve animals the interstitial gland showed a characteristic appearence. The

Effect of Gonadotrophic Stimulation on the Cholesterol Content in the Ovaries of Rats, treated with Prominal. The Right Ovary (R) was removed immediately before the Injection of Pregnant Mare Serum Gonadotrophin and the Left Ovary (L)

Effect of Gonadotrophic Stimulation on the Cholesterol Content in the Ovaries of Rats, treated with Prominal. The Right Ovary (R) was removed immediately before the Injection of Pregnant Mare Serum Gonadotrophin and the Left Ovary (L) removed 48 hours later.

Animal No.	Vaginal Smear Stage at the 1st Day of Treatment	Daily Treatment			Vaginal Smears		Gonadotrophic Stimulation PMSG Hours	Interstitial Gland Schultz Reaction
		Period Days	Dose Mg.		Days Negative	Days Positive		
			Average	Range				
21	Oestrus	10	22.1	9.0–31.5	6	4	100 I. U. 48 Hours	R ++++ L ++, +++
23	Dioestrus	10	20.5	9.0–27.0	2	8	100 I. U. 48 Hours	R ++++, +++++ L ++
26	Prooestrus	10	21.8	9.0–27.0	6	4	100 I. U. 48 Hours	R ++++ L +, ++
41	Oestrus	7	25.2	18.0–31.5	1	6	100 I. U. 48 Hours	L ++++ R 0, +, ++
42	Oestrus	13	26.1	18.0–36.0	9	4	150 I. U. 48 Hours	R ++++ L 0, +, ++
44	Oestrus	7	24.1	9.0–36.0	4	3	100 I. U. 48 Hours	L ++++ R 0, (+)
45	Prooestrus	11	24.3	13.5–31.5	10	1	100 I. U. 48 Hours	R ++++ L 0, +
46	Oestrus	12	22.4	13.5–27.0	10	2	150 I. U. 48 Hours	R ++++ L 0, +
47	Dioestrus	7	24.6	18.0–36.0	3	4	100 I. U. 48 Hours	L +++++ R 0, +, ++
50	Oestrus	11	23.6	13.5–36.0	9	2	150 I. U. 48 Hours	R ++++ L 0, +
51	Oestrus	12	23.1	13.5–36.0	11	1	150 I. U. 48 Hours	R ++++ L 0, +

gland cells were clearly poor in cytoplasm and had small, dense nuclei. No. 2 and 21, presented an interstitial gland of different appearance. The cells hade here more abundant cytoplasm and larger, less compact nuclei. The animal No. 23 showed almost the same picture.

Concerning the other ovarian structures no certain differences could be observed between the ovaries in the various animals. Corpora lutea, growing and Graafian follicles were present in all the ovaries. On the basis of the appearence of corpora lutea it was not possible to decide, if any of these were derived from ovulations during the period of Prominal treatment. There were, however, no recently ruptured follicles in any of the ovaries.

The effects of Prominal treatment as regards the vaginal cycle, uterus and the interstitial cells are summarized in Table III.

TABLE III

Animal No.	3	6	11	17	18	20	26	8	9	2	21	23
Vaginal Reaction	–	–	–	(–)	(–)	(–)	(–)	+	+	+	(–)	+
Uterus	–	–	—	–	–	–		+	+	+		
Interstitial Gland Cells	–	–	–	–	–	–	–	–	–	+	+	+

Vaginal Reaction
+ denotes constant or predominant oestrus.
– denotes constant dioestrus.
(–) denotes dioestrus or alternating dioestrus and oestrus.

Uterus
+ denotes fluid distension and hyperaemia.
— denotes no distension or hyperaemia.

Interstitial Gland Cells
+ denotes larger, less compact nuclei.
— denotes smaller, dense nuclei.

As is evident from Table I all the ovaries, which had not been stimulated with PMSG, showed a pronounced storage of cholesterol as demonstrated by the Schultz reaction. This reaction in the interstitial gland cells was of such a high intensity as is not reached even in the stages of prooestrus or metoestrus within the normal oestrous cycle. Concerning the cholesterol

storage no certain differences could be observed between the ovaries in the animals with persistent oestrus and the ovaries in the rats with constant negative vaginal smears. — Owing to the difficulty of differentiating the theca interna cells from the interstitial gland, the cholesterol content of these cells could not be judged with certainty.

The effect of pregnant mare serum gonadotrophin on the cholesterol, stored in the interstitial cells during the Prominal treatment was examined in eleven animals, No. 21—51. A dose of 100—150 I.U. PMSG, giving a depletion of the stored cholesterol in hypophysectomized rats (Aldman, Claesson, Hillarp and Odeblad, 1949 a), gave an almost complete depletion as is shown in Table II. Thus even a prolonged Prominal treatment does not prevent the cholesterol reduction in the interstitial gland cell at gonadotrophic stimulation. Since this reduction is an essential and characteristic reaction, Prominal seems not to have any toxic side-effect on the interstitial cell with a prevention of its normal ability of reacting on an existing gonadotrophic stimulation. Moreover, from the works of Everett, Markee and Sawyer it is evident that for a short time the barbiturates do not have any toxic effects on the rat ovary.

Discussion

In a previous work (Aldman, Claesson, Hillarp and Odeblad, 1949 a) it has been shown that the storing of cholesterol in the interstitial ovarian cells does not take place as an autonomous cellular process and that small amounts of human chorionic and pregnant mare serum gonadotrophin in combination produce a pronounced storage. In another work (1949 b) it was shown that oestradiol and progesterone, parenterally administered, have the same effect, in all probability by a depression of the pituitary gonadotrophin secretion to a low level. On the basis of these results it was assumed that the cholesterol storing process in these cells is directly regulated by gonadotrophic hormone. The present investigation was carried out in order to support this assumption by further experiments on the effect of low level

gonadotrophin secretion on the cholesterol storage under quite different experimental conditions.

The observations in the present investigation agree with EVERETT, MARKEE and SAWYER's demonstration of a blocking effect by barbiturate on the cyclic release of pituitary gonadotrophin. This is evident from the fact that in no case any recently ruptured follicles were observed in the ovaries and that all animals treated with Prominal for more than 8 days showed a cessation of the vaginal cycle. As in WESTMAN's investigation (1947) two quite different types of persistent vaginal stages were obtained, oestrus and dioestrus respectively. Concerning the animals with persistent dioestrus, the complete lack of signs of oestrogenic influence on the vaginal epithelium indicates that not only has the ovulatory discharge of gonadotrophins been prevented, but also that the gonadotrophin level was very low.[1]

The appearance of the interstitial gland cells still more strongly speaks for a depressant action of Prominal on the pituitary secretion of gonadotrophins. These cells show a picture of almost as high inactivity as the deficiency cells in the ovary of hypophysectomized rats. This indicates that the secretion of interstitial cell stimulating hormone (ICSH) is depressed to a very low level. This pituitary hormone has a specific effect on the cytology of the interstitial gland cells and small amounts of biologically pure ICSH have been shown to repair the deficiency cells (JENSEN, SIMPSON, TOLKSDORF and EVANS, 1939, EVANS, SIMPSON, TOLKSDORF and JENSEN, 1939, SHEDLOVSKY, ROTHEN, GREEP, van DYKE and CHOW, 1940, FRAENKEL-CONRAT, LI, SIMPSON and EVANS, 1940, GREEP, van DYKE and CHOW, 1942, SIMPSON, LI and EVANS, 1942).

The occurrence of persistent oestrus in five of the Prominal treated animals apparently argues against the assumption of a depressant action of this barbiturate on the pituitary secretion of gonadotrophic hormones. A further objection is the fact that the uterus of these animals at the time of autopsy gave evidence

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[1] Of course it cannot be quite excluded that possibly in one single rat of the animals with persistent dioestrus pseudopregnancy was elicited by vaginal stimulation at taking smears.

of oestrogenic secretion. These two findings, however, are no real objections for the following reasons:

a) the vaginal and uterine responses to oestrogens are caused already by amounts on the μg.-level.
b) the interstitial gland cells in two of the animals with persistent oestrus showed the same appearence of inactivity as all the animals with constant dioestrus.
c) EVERETT, MARKEE and SAWYER have shown that a continous oestrogen secretion may be present in spite of a depression of the pituitary gonadotrophin secretion by barbiturates to a subovulatory level.

Thus the fact that certain animals had persistent oestrus and uterine signs of oestrogen secretion admits the possibility of a low secretion of pituitary gonadotrophins. None of the observations in this investigation speaks against such an interpretation. The present material however, does not seem to justify any conclusions concerning the reason why Prominal treatment produces persistent oestrus in certain animals.

The produced evidence gives a high probability to the assumption that the cyclically recurring influences of the hypophysis on the ovary have been prevented during Prominal treatment and that the gonadotrophin secretion in the experimental animals has been on a low level. It is also evident from the investigation that all the ovaries of these animals showed two striking features, a) a pronounced storage of cholesterol in the interstitial cells, and b) a cholesterol storage quite independent of at what time during the treatment the ovaries were removed for examination. Thus the changes in the cholesterol content, normally occurring during the oestrous cycle, have been prevented by the barbiturate sedation. These findings are in full agreement with our previous investigations on the storage mechanism of cholesterol and may plainly be explained on the basis of the assumption that the storage process is directly regulated by gonadotrophic hormone, a low level giving a storage and a high level (of the same or a different gonadotrophin) a reduction of cholesterol in the interstitial cells.

Summary

Adult female rats have been treated with N-methylethylphenylbarbituric acid (Prominal) during 5—15 days. Evidence is presented indicating that this treatment has a depressant effect on the pituitary function, giving a low, non-cyclic gonadotrophic secretion. Under this condition there is a pronounced storage of cholesterol in the ovarian interstitial gland cells. This gives complementary evidence to the assumption of a gonadotrophic regulation of the cholesterol storage in these cells.

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The Effect of Colchicine on the Increase in Ovarian Weight and Phospholipids at Gonadotrophic Stimulation[1]

By

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At gonadotrophic stimulation with chorionic and pregnant mare serum gonadotrophin the rabbit ovary displays a considerable increase both in weight and in phospholipid content (CLAESSON, DICZFALUSY, HILLARP and HÖGBERG, 1948; CLAESSON, HILLARP and HÖGBERG, 1953). It was not possible to decide whether this is an expression of an increase in the number of the interstitial gland cells. As pointed out previously (1948), a solution of this problem is necessary for the calculations of the quantitative changes of the lipids in the stimulated cell. An investigation was therefore carried out on the effect of gonadotrophic stimulation on the ovary, cell division being prevented by means of colchicine.

Material and Methods

The experiments were carried out in the same way as in a previous study (1953). Female rabbits on the 12th day of pseudopregnancy were used. One of the ovaries was removed immediately before the intravenous injection of 450 I.U. pregnant mare serum gonadotrophin — PMSG (Antex Leo[2]), and the other ovary nine and a half hours later. In addition, the animals

[1] The investigation was aided by a grant from the Faculty of Medicine, University of Lund.

[2] A.B. Leo, Helsingborg, put this preparation at our disposal through the kind intermediation of Mr. S. Wiström.

received colchicine (2 mg. per Kg. body weight subcutaneously) 7 hours before the removal of the second ovary. The interval between the administration of the PMSG and the colchicine was fixed at two and a half hours as neither the increase in weight or in phospholipids occur until after 3 hours following stimulation with this dose of PMSG (CLAESSON, HILLARP and HÖGBERG, 1953). The dose of colchicine, 2 mg. per Kg., is sufficient to arrest the mitosis for 7 hours (DUSTIN, 1934; LUDFORD, 1936).

In the experiments 17 animals were used. The ovaries were examined histologically in 3 animals. They were fixed in 10 per cent formalin (kept over $CaCO_3$), embedded in paraffin, sectioned (10μ) and stained with haematoxylin-eosin. Determinations of cholesterol and phospholipids were carried out on the ovaries of the other animals in the same way as reported in previous works (1948, 1953).

Results

The gonadotrophically stimulated, colchicine treated ovaries showed numerous arrested mitotic figures in the granulosa layer and to a lesser extent in the theca interna of the growing follicles. The interstitial gland, on the other hand, showed only sparse occurrence of such figures. Otherwise the interstitial gland exhibited the usual picture at strong gonadotrophic stimulation, viz. cells of a swollen appearance, with abundant cytoplasm and large, lightly stained nuclei.

The rest of the material is presented in Tables I and II where the changes in ovarian weight and lipid content of every particular animal is expressed in mg. and per cent. The experiment reported in the present paper was carried out immediately after the quantitative investigations of the time course of the ovarian changes at stimulation with PMSG (1953) and in the same manner (apart from the cholchicine treatment). The results of these two investigations have therefore been compared and grouped together in Tables III and Fig. 1.

The increase in ovarian weight in the colchicine treated animals was 42.1 ± 4.4 per cent or 94.3 ± 16.5 mg. nine and a half hours following the administration of PMSG. The phospholipid

TABLE I.

Changes in the ovarian weight of pseudo-pregnant rabbits at gonadotrophic stimulation. The first ovary extirpated immediately before the intravenous administration of 450 I.U. PMSG. After the elapse of 2½ hours the animals were treated with Colchicine 2 mg./kg. subcutaneously. The other ovary removed 9½ hours after the injection of PMSG and 7 hours after treatment with Colchicine.

Animal No.	Weight of the Ovaries		
	Mg.	Changes	
		Mg.	Per cent
1	L 140 R 218	+ 78	+55.7
2	L 175 R 240	+ 65	+37.1
3	L 118 R 171	+ 53	+44.9
4	L 233 R 393	+160	+68.7
5	L 125 R 202	+ 77	+61.6
6	L 304 R 449	+145	+47.7
7	L 411 R 525	+114	+27.7
9	L 130 R 194	+ 64	+49.2
10	L 585 R 712	+127	+21.7
11	L 150 R 150	± 0	± 0
12	L 132 R 169	+ 37	+28.0
13	L 459 R 747	+288	+62.7
14	L 182 R 244	+ 62	+34.1
15	L 291 R 400	+109	+37.5
16	L 143 R 205	+ 62	+43.4
17	L 124 R 191	+ 67	+54.0
M.=		+ 94.3	+42.1
S.E.=		± 16.5	± 4.4
S.D.=		± 65.9	±17.6
n=		16	16

TABLE II.

Lipid changes in the ovarian interstitial gland at gonadotrophic stimulation. The first ovary extirpated immediately before the intravenous administration of 450 I.U. PMSG. After the elapse of 2 1/2 hours the animals were treated with Colchicine 2 mg./kg. subcutaneously. The other ovary removed 9 1/2 hours after the injection of PMSG and 7 hours after treatment with Colchicine.

Animal No.	Weight of Ovaries	Total Cholesterol			Free Cholesterol			Esterified Cholesterol			Phospholipids		
		Mg./g.	Changes		Mg./g.	Changes		Mg./g.	Changes		Mg./g.	Changes	
			Mg./g.	Per cent		Mg./g.	Per cent		Mg./g.	Per cent		Mg./g.	Per cent
1	L 140												
2	L 175	18.4											
1	R 218												
2	R 240	7.6	−10.8	−58.7									
3	L 118												
5	L 125	16.7											
3	R 171												
5	R 202	2.6	−14.1	−84.4									
4	L 233	21.5											
4	R 393	12.1	− 9.4	−43.7									
6	L 304	17.2			2.8			14.4			28.7		
6	R 449	5.4	−11.8	−68.6	1.7	−1.1	−39.3	3.7	−10.7	−74.3	34.0	+ 5.3	+18.5
7	L 411	27.0			3.3			23.7			26.0		
7	R 525	10.5	−16.5	−61.1	2.9	−0.4	−12.1	7.6	−16.1	−67.9	31.4	+ 5.4	+20.8
10	L 585	24.8			2.8			22.0			28.9		
10	R 712	9.7	−15.1	−60.9	1.9	−0.9	−32.1	7.8	−14.2	−64.5	32.7	+ 3.8	+13.1
11	L 150												
12	L 132	23.9			3.9			20.0			23.2		

7	R 525	10.5	−16.5	−61.1	2.9	−0.4	−12.1	7.6	−16.1	−67.9	31.4	+ 5.4	+20.8
10	L 585	24.8			2.8			22.0			28.9		
10	R 712	9.7	−15.1	−60.9	1.9	−0.9	−32.1	7.8	−14.2	−64.5	32.7	+ 3.8	+ 13.1
11	L 150												
12	L 132	23.9			3.9			20.0			23.2		
11	R 150												
12	R 169	9.8	−14.1	−60.0	3.4	−0.5	−12.8	6.4	−13.6	−68.0	25.3	+ 2.1	+ 9.1
13	L 459	37.6			—			—			28.1		
13	R 747	14.9	−22.7	−60.4	1.0	—	—	13.9	—	—	39.9	+11.8	+42.0
14	L 182												
16	L 143	19.3			3.8			15.5			20.7		
14	R 244												
16	R 205	13.2	− 6.1	−31.6	3.4	−0.4	−10.5	9.8	− 5.7	−36.8	26.9	+ 6.2	+30.0
15	L 291	29.4			4.3			25.1			23.8		
15	R 400	11.6	−17.8	−60.5	3.5	−0.8	−18.6	8.1	−17.0	−67.7	30.9	+ 7.1	+29.8
	M.=		−13.1	−58.9		−0.6	−18.6		−12.1	−60.5		+ 5.6	+22.5
	S.E.=		± 1.2	± 4.1		±0.1	± 3.9		± 1.5	± 5.3		± 1.0	± 3.7
	S.D.=		± 4.5	±15.5		±0.3	±11.0		± 4.3	±14.9		± 2.9	±11.2
	n=		14			8			8			9	

TABLE III.

Changes of the lipids in the ovarian interstitial gland and of the ovarian weight[1] *at gonadotrophic stimulation (pseudopregnant rabbits). The first ovary exstirpated immediately before the intravenous administration of 450 I.U. PMSG, the other one removed 1 1/2, 3, 6, 9 1/2 and 12 hours respectively after the injection of the hormone. In addition the cholchicinetreated animals received 2 mg. colchicine/kg. in 0.9 per cent NaCl subcutaneously. The values of 1 1/2, 3, 6 and 12 hours derive from a previous material* (CLAESSON, HILLARP and HÖGBERG 1953).

Gonadotrophic Stimulation Hours	Weight of Ovaries	Total Cholesterol	Free Cholesterol	Esterified Cholesterol	Phospho-lipids	Total Fatty Acids	Residual Fatty Acids
1 1/2	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+4.2± 6.7 S.D. = ±16.4	−5.9±1.6 S.D. = ±3.9	+0.17±0.17 S.D. = ±0.41	−6.1±1.6 S.D. = ±3.8	+1.9±1.4 S.D. = ±3.3	−3.5±2.1 S.D. = ±5.2	−0.83±2.6 S.D. = ±6.5
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	+1.3±2.7 S.D. = ±6.6	−13.7±3.8 S.D. = ±9.4	+3.5± 4.5 S.D. = ±11.1	−15.2±4.0 S.D. = ±9.8	+6.2± 4.3 S.D. = ±10.5	−4.1±3.0 S.D. = ±7.3	+0.9± 9.0 S.D. = ±22.1
	n=6	n=6	n=6	n=6	n=6	n=6	n=6
3	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+9.4± 7.1 S.D. = ±21.4	−12.2±1.1 S.D. = ±3.2	−0.44±0.6 S.D. = ±1.8	−11.8±1.1 S.D. = ±3.3	+0.2±0.35 S.D. = ±1.05	−7.2±1.6 S.D. = ±4.8	+0.4±1.0 S.D. = ±2.9
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	+2.4± 3.6 S.D. = ±10.7	−41.0±1.9 S.D. = ±5.7	−12.7±3.0 S.D. = ±9.1	−44.6±2.2 S.D. = ±6.6	+0.8±1.4 S.D. = ±4.3	−10.9±2.4 S.D. = ±7.1	+0.6±3.1 S.D. = ±9.2
	n=9	n=9	n=9	n=9	n=9	n=9	n=9
6	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+69.8± 13.3	−24.8±1.7	+1.0±0.91	−23.0±1.7	+2.8±0.89	−15.2±2.1	−1.2±1.1

	+2.4± 3.6 S.D.=±10.7	−41.0±1.9 S.D.=±5.7	−12.7±3.0 S.D.=±9.1	−44.6±2.2 S.D.=±6.6	+0.8±1.4 S.D.=±4.3	−10.9±2.4 S.D.=±7.1	+0.6±3.1 S.D.=±9.2
	n=9	n=9	n=9	n=9	n=9	n=9	n=9
6	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+69.8± 13.2 S.D.=± 45.8	−24.8±1.7 S.D.=±5.9	−1.0±0.01 S.D.=±0.42	−23.9±1.7 S.D.=±6.0	+2.8±0.38 S.D.=±1.3	−15.2±2.1 S.D.=±7.2	−1.2±1.1 S.D.=±3.7
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	+25.2± 5.0 S.D.=±17.2	−64.9±1.7 S.D.=±6.0	−25.3±2.7 S.D.=±9.4	−69.3±2.0 S.D.=±6.8	+9,7±1.2 S.D.=±4.3	−20.9±2.1 S.D.=±7.3	−2.4± 3.7 S.D.=±12.8
	n=12	n=12	n=12	n=12	n=12	n=12	n=12
9 1/2 Colchicinetreated Animals	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+94.3±16.5 S.D.=±65.9	−13.1±1.2 S.D.=±4.5	−0.6±0.1 S.D.=±0.3	−12.1±1.5 S.D.=±4.3	+5.6±1.0 S.D.=±2.9		
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	+42.1±13.9 S.D.=±55.6	−58.9± 4.1 S.D.=±15.5	−18.6± 3.9 S.D.=±11.0	−60.5± 5.3 S.D.=±14.9	22.5± 3.7 S.D.=±11.2		
	n=16	n=14	n=8	n=8	n=8		
12	Mg.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.	Mg./g.
	+140.4± 14.0 S.D.=± 39.7	−26.0±1.1 S.D.=±3.0	−1.1±1.3 S.D.=±3.6	−25.0±0.92 S.D.=±2.6	+8.5±1.0 S.D.=±2.9	−25.0± 8.0 S.D.=±22.6	−14.1±2.4 S.D.=±6.8
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	+48.4± 5.4 S.D.=±15.3	−72.4±2.5 S.D.=±7.1	−27.5±2.9 S.D.=±8.2	−77.8±2.5 S.D.=±7.2	+31.9± 3.8 S.D.=±10.8	−28.4±1.1 S.D.=±3.0	−29.0±1.8 S.D.=±5.1
	n=8	n=8	n=8	n=8	n=8	n=8	n=8

[1] Mean ± Standard Error S.D. = Standard Deviation n = No. of observations

content in the stimulated ovaries increased 22.5 ± 3.7 per cent or 5.6 ± 0.97 mg./g. ovarian tissue, if the calculation is made on the basis of the weight of the unstimulated ovaries. As appears from Fig. 1, these mean values are practically lying on the line connecting the mean values of the increase in phospholipids and in ovarian weight in the non colchicine-treated animals after 6 and 12 hours of stimulation, respectively.

The changes in the cholesterol content of the stimulated ovaries were studied in order to establish whether the colchicine interfered with the reduction in esterified cholesterol, normally occuring at gonadotrophic stimulation. The observed reduction in this fraction was somewhat smaller (60.5 ± 5.3 per cent) than that found in the non colchicine-treated animals. The gonadotrophic stimulation had full effect, however, in spite of the cholchicine treatment as is shown by the finally obtained values of the cholesterol content: they were reduced to the same low level as in the non cholchicine-treated animals (Table IV). The fact that the decrease in cholesterol expressed in per cent or mg./g. does not reach higher values must consequently be due to the comparatively low content of cholesterol esters present before the stimulation (Table IV).

TABLE IV

The content of esterified cholesterol in the ovaries before and after stimulation with 450 I.U. pregnant mare serum gonadotrophin.

Gonadotrophic stimulation Hours	6	Colchicine treated animals $9\frac{1}{2}$	12
M_F	31.5 ± 2.4 S.D. $= \pm 8.1$	19.5 ± 1.4 S.D. $= \pm 4.0$	32.1 ± 1.1 S.D. $= \pm 3.0$
M_E	10.6 ± 0.89 S.D. $= \pm 3.1$	7.5 ± 0.71 S.D. $\pm = 2.0$	7.2 ± 0.81 S.D. $= \pm 2.3$
Number of animals	12	8	8

M_F = Mean mg./g. before PMSG.
M_E = Mean mg./g. after PMSG.

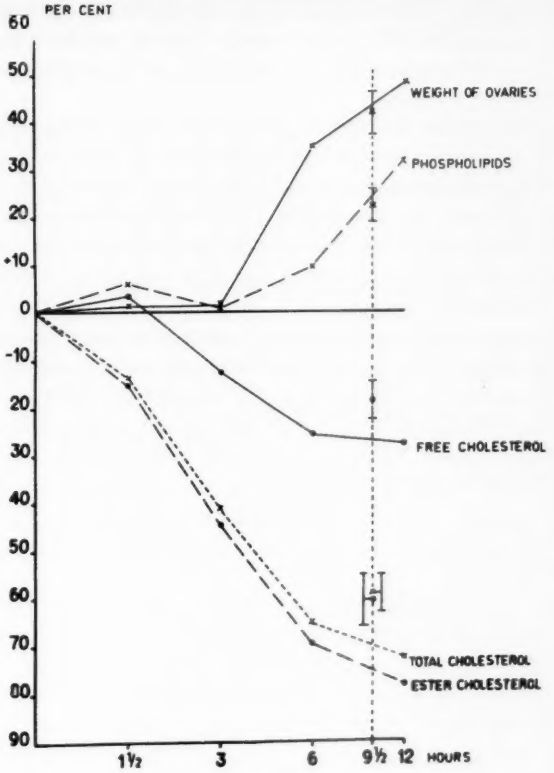

Fig. 1.

Discussion

The colchicine technique has proved very useful in studies on the growth of various structures in the ovary (ALLEN, SMITH and GARDNER, 1936, 1937; LANE and DAVIS, 1939, SCHMIDT, 1942; ALLEN, THOMAS, WILSON and HESSION, 1943). According to the results reported in these works and by other authors as well (e. g. DUSTIN, 1934; LUDFORD, 1936), the dose of colchicine used in the present experiment, 2 mg. per Kg. body weight, should be quite sufficient to arrest the mitosis in the ovary during 7 hours of

gonadotrophic stimulation. The histological examination showed, moreover, abundant arrested mitotic figures in the granulosa layer of the developing follicles which has a rapid growth under the influence of PMSG.

It appears from the investigation that PMSG brings forth an increase in weight and phospholipids also in colchicine treated ovaries. Compared to the animals examined immediately before this experiment, the increase is lying within the normal range of the non colchicine-treated rabbits. Therefore, these effects of PMSG on the ovaries are not a result of a cellular proliferation in the interstitial gland.

It is conceivable, however, that colchicine may prevent the effect of PMSG on the interstitial cells. If so, this might invalidate the conclusion made above. The experiments show, however, that PMSG produces a normal reduction of the esterified cholesterol stored in the interstitial cells also in colchicine treated ovaries. Since this reduction is an essential and characteristic reaction of the gonadotrophically stimulated gland cell, the colchicine cannot have seriously interfered with the stimulation of this cell.

In earlier investigations the marked increase in weight of the ovary at gonadotrophic stimulation gave rise to difficulties in calculating the lipid changes in the ovary. It is quite evident that in order to obtain a definite information about the real changes within the individual cell at stimulation, one must be able to exclude the possibility of a multiplication of cells during the course of the experiment. The present investigation shows that there is no increase in the number of cells at stimulation with PMSG under the experimental conditions used or if any, that it is of no importance.

Summary

The effect of pregnant mare serum gonadotrophin (450 I.U. PMSG) on the ovarian interstitial gland of pseudopregnant rabbits treated with colchicine was investigated.

Ovarian weight, phospholipids and the stored esterified cholesterol were shown to undergo normal changes.

The present investigation shows that there is no increase in the number of cells at stimulation with PMSG under the experimental conditions given or if any, that it is of no importance.

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From the Department of Histology, Lund Sweden

Quantitative Relationship between Gonadotrophic Stimulation and Lipid Changes in the Interstitial Gland of the Rabbit Ovary[1]

By

LENNART CLAESSON

In previous studies on the lipid metabolism in the interstitial gland of the rabbit ovary at gonadotrophic stimulation, considerable quantitative changes in the lipids were reported (CLAESSON, DICZFALUSY, HILLARP, and HÖGBERG, 1948). An examination of the time course of these lipid changes (CLAESSON, HILLARP and HÖGBERG, 1953) showed that a pronounced reduction in the stored cholesterol esters occurred during the acute phase of the gonadotrophic stimulation as well as a considerable reduction in the fatty acids in other fractions. The time relation between the changes in the cholesterol esters and in the other lipid fractions seemed to indicate that the latter were somehow linked up with the processes which in the first place cause a reduction in the cholesterol esters in the ovarian interstitial gland at an acute supramaximal gonadotrophic stimulation. It seemed necessary, therefore, to clarify the quantitative relationship between the dose of injected gonadotrophic hormone and the degree of reduction in ovarian cholesterol esters and also the connexion of the cholesterol decrease with other lipid changes. An investigation dealing with these problems is presented in this paper.

Material and Methods

In order to keep the experimental conditions as constant as possible, all the experimental animals were treated uniformly,

[1] Aided by a grant from the Faculty of Medicine, University of Lund.

TAB

Control animals. The first ovary extirpated immediately before ...*ravene*
moved 12 h...*er the*

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholesterol			Es... Cho...
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Changes		
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	Per ce...	...g. M
70	L 136 R 170	+34	+25.0	24.0 31.8	+ 7.8	+32.5	3.7 5.5	+1.8	+4...	20.3 26.3 +
71	L 402 R 428	+26	+ 6.5	41.8 43.9	+ 2.1	+ 5.0	2.7 3.0	+0.3	+1...	39.1 40.9 +
72	R 262 L 262	± 0	± 0	28.4 30.0	+ 1.6	+ 5.6	2.6 2.5	—0.1	— ...	25.8 27.5 +
73	R 180 L 192	+12	+ 6.7	34.3 32.5	— 1.8	— 5.2	3.0 3.7	+0.7	+2...	31.3 28.8 —
74	L 148 R 155	+ 7	+ 4.7	36.5 43.6	+ 7.1	+19.5	3.6 4.2	+0.6	+1...	32.9 39.4 +
75	R 288 L 205	—83	—28.8	23.5 19.6	— 3.9	—16.6	2.3 2.0	—0.3	—1...	31.2 27.6 —
76	L 197 R 176	—21	—10.7	26.6 22.9	— 3.7	—13.9	3.0 2.8	—0.2	— ...	33.6 30.1 —
77	R 217 L 209	— 8	— 3.7	26.0 25.3	— 0.7	— 2.7	3.2 3.0	—0.2	— ...	32.8 32.3 —
78	L 181 R 256	+75	+41.4	26.7 37.0	+10.3	+38.6	3.2 4.2	+1.0	+3...	33.5 42.8 +
79	R 435 L 412	—23	— 5.3	40.4 35.2	— 5.2	—12.9	2.9 2.7	—0.2	— ...	37.5 32.5 —
80	L 275 R 274	— 1	— 0.4	27.6 19.9	— 7.7	—27.9	— 2.7	—	— ...	— 31.2
	M.=	+ 1.6	+ 3.2		+ 0.5	+ 2.0		+0.3	+ ...	+
	S.E.=	±11.9	± 5.5		± 1.8	± 6.3		±0.2	± ...	±
	S.D.=	±39.4	±18.2		± 5.8	±21.0		±0.7	±2...	±
	n=	11			11			10		

...venous administration of 2 ml 0.9 per cent NaCl, the other one re... the injection.

Esterified Cholesterol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
	Changes			Changes			Changes			Changes	
Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
10.3 6.3	+6.0	+29.6	23.2 28.5	+ 5.3	+22.8	— —			— —	—	—
9.1 0.9	+1.8	+ 4.6	31.0 32.6	+ 1.6	+ 5.2	91.4 106.4	+15.0	+16.4	44.7 57.4	+12.7	+28.4
5.8 7.5	+1.7	+ 6.6	29.0 29.5	+ 0.5	+ 1.7	52.9 46.3	− 6.6	−12.5	16.4 8.3	− 8.1	−49.4
1.3 8.8	−2.5	− 8.0	— 29.0	—	—	67.9 69.4	+ 1.5	+ 2.2	— 30.9	—	—
2.9 9.4	+6.5	+19.8	24.8 26.7	+ 1.9	+ 7,7	98.3 96.8	− 1.5	− 1.5	59.8 52.7	− 7.1	−11.9
1.2 7.6	−3.6	−17.0	24.8 20.4	− 4.4	−17.7	59.2 61.6	+ 2.4	+ 4.1	28.6 36.3	+ 7.7	+26.9
3.6 0.1	−3.5	−14.8	27.8 26.1	− 1.7	− 6.1	77.6 69.1	− 8.5	−11.0	43.3 38.3	− 5.0	−11.5
2.8 2.3	−0.5	− 2.2	23.7 22.1	− 1.6	− 6.8	67.0 62.3	− 4.7	− 7.0	36.0 32.7	− 3.3	− 9.2
3.5 2.8	+9.3	+39.6	24.2 36.2	+12.0	+49.6	73.7 107.4	+33.7	+45.7	41.9 61.4	+19.5	+46.5
7.5 2.5	−5.0	−13.3	25.8 24.5	− 1.3	− 5.0	87.0 87.8	+ 0.8	+ 0.9	44.8 49.8	+ 5.0	+11.2
— 1.2	—	—	26.2 29.1	+ 2.9	+11.1	69.4 83.3	+13.9	+20.0	— 52.4	—	—
	+1.0	+ 4.5		+ 1.5	+ 6.3		+ 4.6	+ 5.7		+ 2.7	+ 3.9
	±1.6	± 6.2		± 1.5	± 6.0		± 4.1	± 5.6		± 3.6	±10.8
	±4.9	±19.6		± 4.6	±18.9		±12.9	±17.6		±10.2	±30.5
	10			10			10			8	

TA

Animals stimulated with 5 I.U. PMSG. The first ovary exstirp
other one remove

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholester		
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Change	
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	P ce
10	L 297 R 250	−47	−15.8	40.6 37.4	− 3.2	− 7.9	3.6 2.9	−0.7	−1
14	L 226 R 265	+39	+17.3	49.9 49.9	± 0	± 0	3.4 4.7	+1.3	+3
18	L 197 R 248	+51	+25.9	47.1 59.1	+12.0	+25.5	3.9 5.0	+1.1	+2
26	R 220 L 272	+52	+23.6	40.9 41.4	+ 0.5	+ 1.2	3.0 4.2	+1.2	+4
32	L 338 R 363	+25	+ 7.4	41.6 46.2	+ 4.6	+11.1	3.1 3.2	+0.1	+
46	R 305 L 324	+19	+ 6.2	38.5 42.7	+ 4.2	+10.9	3.4 3.6	+0.2	+
52	R 380 L 379	− 1	− 0.3	48.5 40.4	− 8.1	−16.7	3.2 3.0	−0.2	−
63	L 201 R 216	+15	+ 7.5	20.0 19.3	− 0.7	− 3.5	2.4 2.5	+0.1	+
64	L 191 R 229	+38	+19.9	37.2 37.6	+ 0.4	+ 1.1	3.4 3.8	+0.4	+1
69	L 238 R 260	+22	+ 9.2	26.7 25.3	− 1.4	− 5.4	3.1 2.8	−0.3	−
	M.=	+21.3	+ 8.8		+ 0.8	+ 1.6		+0.3	+
	S.E.=	± 9.2	± 2.8		± 1.7	± 1.2		±0.2	±
	S.D.=	±29.1	± 8.8		± 5.3	± 3.7		±0.7	±2
	n=	10			10			10	

mediately before the intravenous administration of the hormone, the urs after the injection.

Esterified Cholesterol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
	Changes			Changes			Changes			Changes	
Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
37.0 34.5	− 2.5	− 6.8	21.0 17.7	−3.3	−15.7	79.8 52.5	−27.3	−34.2	41.1 17.7	−23.4	− 56.9
46.5 45.2	− 1.3	− 2.8	25.1 33.2	+8.1	+32.3	81.2 102.7	+21.5	+26.5	33.5 50.4	+16.9	+ 50.4
43.2 54.1	+10.9	+25.2	— —	—	—	84.7 82.3	− 2.4	− 2.8	— —	—	—
57.9 57.2	− 0.7	− 1.8	— —	—	—	72.7 95.5	+22.8	+31.4	— —	—	—
38.5 43.0	+ 5.1	+11.7	— —	—	—	88.9 113.3	+24.4	+27.4	— —	—	—
35.1 39.1	+ 4.0	+11.3	24.6 29.0	+4.4	+17.9	106.6 106.6	± 0	± 0	66.8 61.2	− 5.6	− 8.4
45.3 37.4	− 7.9	−17.4	24.6 26.9	+2.3	+ 9.3	102.0 111.8	+ 9.8	+ 9.6	55.4 68.9	+13.5	+24.4
17.6 16.8	− 0.8	− 4.5	23.2 26.2	+3.0	+12.9	56.9 62.1	+ 5.2	+ 9.1	29.7 33.4	+ 3.7	+12.5
33.8 33.8	± 0	± 0	23.0 29.3	+6.3	+27.4	87.3 127.4	+40.1	+45.9	49.4 85.5	+36.1	+ 73.1
23.6 22.5	− 1.1	− 4.7	25.2 29.1	+3.9	+15.5	73.1 83.3	+10.2	+14.0	40.6 48.9	+ 8.3	+20.4
	+ 0.6	− 1.0		+3.5	+14.2		+10.4	+12.7		− 7.1	+16.5
	± 1.6	± 3.8		±1.3	± 5.9		± 5.9	± 7.1		± 7.0	±15.8
	± 5.1	±12.0		±3.5	±15.5		±18.5	±22.3		±18.6	± 41.7
	10			7			10			7	

TA

Animals stimul *h 10 I*

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholesterol			Este Chol	
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Changes		g./g.	M
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	Pe cen		
11	L 297 R 268	−29	− 9.8	33.4 25.4	− 8.0	−24.0	2.7 4.9	+2.2	+8	30.7 20.5	−
15	L 263 R 202	−61	−23.2	48.0 11.5	−36.5	−76.0	4.6 3.7	−0.9	−1	43.4 7.8	−
19	L 251 R 215	−36	−14.3	48.5 39.8	− 8.7	−17.9	2.9 3.6	+0.7	+2	45.6 36.2	−
22	R 358 L 353	− 5	− 1.4	39.1 32.5	− 6.6	−16.9	3.4 3.2	−0.2	−	35.7 29.3	−
27	R 443 L 469	+26	+ 5.9	34.3 42.8	+ 8.5	+24.8	3.4 3.4	±0	± 0	30.9 39.4	+
33	L 412 R 402	−10	− 2.4	43.2 47.4	+ 4.2	+ 9.7	4.5 4.0	−0.5	−11	38.7 43.4	+
40	R 412 L 340	−72	−17.5	33.1 27.7	− 5.4	−16.3	3.2 2.6	−0.6	−18	29.9 25.1	−
51	R 338 L 362	+24	+ 7.1	36.2 37.2	+ 1.0	+ 2.8	3.6 3.5	−0.1	−	32.6 33.7	+
61	L 249 R 295	+46	+18.5	– –	–	–	– –	–	–	– –	
62	L 274 R 296	+22	+ 8.0	20.6 10.0	−10.6	−51.5	2.6 2.7	+0.1	+ 4	18.0 7.3	−
68	L 283 R 304	+21	+ 7.4	25.8 21.3	− 4.5	−17.4	2.8 3.1	+0.3	+10	23.0 18.2	−
	M.=	− 6.7	− 1.6		− 6.7	−18.3		+0.1	+ 6		−
	S.E.=	±11.7	± 3.7		± 3.8	± 9.1		±0.3	± 9		±
	S.D.=	±38.7	±12.3		±12.1	±28.9		±0.9	±29		±
	n=	11			10			10			

TA

ls stimul *h 10 I.U. PMSG.*

Cholesterol		Esterified Cholesterol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
Changes			Changes			Changes			Changes			Changes	
Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
+2.2	+8	30.7 20.5	−10.2	−33.2	23.6 18.2	−5.4	−22.7	73.0 56.2	−16.8	−23.0	36.8 30.4	− 6.4	− 17.4
+0.9	−1	43.4 7.8	−35.6	−82.0	23.6 23.8	+0.2	+ 0.8	91.8 57.0	−34.8	−37.1	47.1 35.9	−11.2	− 23.8
+0.7	+2	45.6 36.2	− 9.4	−20.6	25.7 22.2	−3.5	−13.6	87.6 63.7	−23.9	−27.3	40.1 24.8	−15.3	− 38.2
0.2	− 5	35.7 29.3	− 6.4	−17.8	23.5 —	—	—	52.4 73.4	+21.0	+40.1	13.0 —	—	—
0	± 0	30.9 39.4	+ 8.5	+27.5	26.6 30.0	+3.4	+12.8	93.2 86.5	− 6.7	− 7.2	54.9 40.2	−14.7	− 26.8
0.5	−11	38.7 43.4	+ 4.7	+12.1	23.5 31.8	+8.3	+35.3	93.9 96.9	+ 3.0	+ 3.2	52.5 46.8	− 5.7	− 10.9
0.6	−19	29.9 25.1	− 4.8	−16.1	25.4 21.6	−3.8	−15.0	93.9 89.1	− 4.8	− 5.1	57.0 58.0	+ 1.0	+ 1.8
0.1	− 2	32.6 33.7	+ 1.1	+ 3.4	25.9 —	—	—	96.3 88.7	− 7.6	− 7.9	57.3 —	—	—
—	-	— —	—	—	— —	—	—	— —	—	—	— —	—	—
0.1	+ 3	18.0 7.3	−10.7	−59.4	25.3 27.0	+1.7	+ 6.7	36.8 34.5	− 2.3	− 6.2	7.9 11.6	+ 3.7	+46.9
0.3	+10	23.0 18.2	− 4.8	−20.9	24.1 26.4	+2.3	+ 9.5	52.9 67.7	+14.8	+28.0	21.5 38.0	+16.5	+76.7
0.1	+ 6		− 6.8	−20.7		+0.4	+ 1.7		− 5.8	− 4.3		− 4.0	+ 1.0
0.3	± 9		± 3.8	±10.8		±1.7	± 6.6		± 5.3	± 7.5		± 3.8	± 1.4
0.9	+29		±12.0	±34.2		±4.7	±18.7		±16.8	±23.7		±10.7	± 4.0
10			10			8			10			8	

TAB

Animals stimul *th 20 I.*

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholesterol			Este Chol	
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Changes		Mg./g.	Mg
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	Per cent		
8	L 225 R 288	+ 63	+28.0	31.4 10.2	−21.2	−67.5	4.1 3.0	−1.1	−2	27.3 7.2	−2
12	L 321 R 350	+ 29	+ 9.0	42.5 23.7	−18.8	−44.2	3.4 2.9	−0.5	−14	39.1 20.8	−1
16	L 319 R 331	+ 12	+ 3.8	49.0 14.5	−34.5	−70.4	3.5 2.8	−0.7	−2	45.5 11.7	−3
20	L 286 R 301	+ 15	+ 5.2	57.2 45.8	−11.4	−19.9	3.3 3.2	−0.1	− 3	53.9 42.6	−1
23	R 298 L 368	+ 70	+23.4	65.3 21.3	−44.0	−67.4	4.0 3.1	−0.9	−22	61.3 18.2	−4
28	R 315 L 369	+ 54	+17.1	52.3 23.7	−28.6	−54.7	1.9 2.6	+0.7	+36	50.4 21.1	−2
34	L 348 R 440	+ 92	+26.4	43.6 12.8	−30.8	−70.6	3.3 2.6	−0.7	−21	40.3 10.2	−3
41	R 319 L 344	+ 25	+ 7.8	34.8 26.4	− 8.4	−24.1	3.5 1.7	−1.8	−51	31.3 24.7	−
59	L 257 R 320	+ 63	+24.5	20.1 6.9	−13.2	−65.7	2.7 2.6	−0.1	− 3	17.4 4.3	−1
60	L 286 R 305	+ 19	+ 6.6	22.0 21.4	− 0.6	− 2.7	2.7 2.9	+0.2	+ 7	19.3 18.5	−
65	L 248 R 280	+ 32	+12.9	25.6 23.7	− 1.9	− 7.4	3.9 3.2	−0.7	−17	21.7 20.5	−
66	L 316 R 440	+124	+39.2	30.1 16.1	−14.0	−46.5	2.7 3.2	+0.5	+18	27.4 12.9	−1
	M.=	+49.8	+17.0		−19.0	−45.1		−0.4	− 9		−1
	S.E.=	± 9.9	± 3.3		± 3.8	± 7.3		±0.2	± 6		±
	S.D.=	±34.4	±11,3		±13.3	±25.4		±0.7	±23		±1
	n=	12			12			12			

TAB
stimul[illegible]*th 20 I.U. PMSG.*

holesterol		Esterified Cholestrol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
Changes			Changes			Changes			Changes			Chaeges	
g./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
1.1	−26	27.3 7.2	−20.1	−73.6	17.8 20,7	+ 2.9	+16.3	75.0 50.0	−25.0	−33.3	44.9 31.4	−13.5	−30.1
0.5	−10	39.1 20.8	−18.3	−46.8	24.9 27.3	+ 2,4	+ 9.6	62.3 62.3	± 0	± 0	19.6 30.2	+10.6	+54.1
0.7	−2	45.5 11.7	−33.8	−74.3	— —	—	—	74.5 44.4	−30.1	−40.4	— —	—	—
0.1	− 3	53.9 2.6	−11.3	−21.0	— —	—	—	83.9 70.0	−13.9	−16.6	— —	—	—
0.9	−22	31.3 8.2	−43.1	−70.3	26.0 —	—	—	73.8 55.2	−18.6	−25.2	15.6 —	—	—
0.7	+36	30.4 21.1	−29.3	−58.3	23.5 —	—	—	95.2 79.4	−15.8	−16.6	45.9 —	—	—
0.7	−21	40.3 10.2	−30.1	−74.7	22.3 —	—	—	86.3 86.1	− 0.2	− 0.2	44.6 —	—	—
1.8	−5	31.3 24.7	− 6.6	−21.1	23.0 27.4	+ 4.4	+19.1	90.2 91.4	+ 1.2	+ 1.3	54.0 56.7	+ 2.7	+ 5.0
0.1	−	17.4 4.3	−13.1	−75.3	25.6 34.0	+ 8.4	+32.8	58.0 49.6	− 8.4	−14.5	29.3 24.1	− 5.2	−17.7
0.2	+	39.3 8.5	− 0.8	−41.0	24.8 28.0	+ 3.2	+12.9	40.0 41.3	+ 1.3	+ 3.3	10.6 10.3	− 0.3	− 2.8
0.7	−17	21.7 9.5	− 1.2	−55.0	23.2 29.2	+ 6.0	+25.9	56.1 62.5	+ 6.4	+11.4	26.2 29,4	+ 3.2	+12.2
0.5	+18	37.4 21.9	−14.5	−52.9	26.9 37.4	+10.5	+39.0	73.6 82.0	+ 8.4	+11.4	37.4 48.5	+11.1	+29.7
0.4	− 9		−18.5	−48.2		+ 5.4	+22.2		− 7.9	−10.0		+ 1.2	+ 8.4
0.2	± 6		± 3 8	± 8.1		± 1.2	± 4.1		± 3.7	± 5.2		± 3.3	± 10.7
0.7	±23		±13.3	±28.0		± 3.1	±10.8		±12.7	±17.1		± 8.7	±28.3
12			12			7			12			7	

TAB

Animals stimul *h 40 I*

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholesteri			Est Chol
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Changes		g./g. M
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	Pe ce	
13	L 231 R 355	+124	+53.7	30.2 5.6	−24.6	−81.5	3.4 3.1	−0.3	−	26.8 2.5 —
17	L 296 R 352	+ 56	+18.9	41.8 20.0	−21.8	−52.2	3.8 3.6	−0.2	−	38.0 36.4 —
21	L 189 R 252	+ 63	+33.3	44.3 13.5	−30.8	−69.5	3.3 3.0	−0.3	−	41.0 10.6 —
24	R 215 L 305	+ 90	+41.9	— —	—	—	— —	—	—	— —
35	L 472 R 592	+120	+25.4	34.8 6.9	−27.9	−80.2	2.8 2.2	−0.6	−2	32.0 4.7 —
36	L 385 R 400	+ 15	+ 3.9	47.5 19.3	−28.2	−59.4	2 9 2.5	−0.4	−1	44.6 16.8 —
42	R 465 L 613	+148	+31.8	47.4 14.7	−32.7	−69.0	3.5 3.6	+0.1	+	43.9 11.1 —
50	R 382 L 402	+ 20	+ 5.2	40.2 12.4	−27.8	−69.2	3.9 2.0	−1.9	−4	36.3 10.4 —
55	L 341 R 361	+ 20	+ 5.9	23.9 22.9	− 1.0	− 4.2	3.2 2.9	−0.3	−	20.7 20.0 —
	M.=	+ 72.9	+24.4		−24.4	−60.7		−0.5	−1	—
	S.E.=	± 16.7	± 6.2		± 3.5	± 8.7		±0.2	±	±
	S.D.=	± 50.1	±17.6		±10.0	±24.7		±0.6	±1	±
	n=	9			8			8		

TAB

ils stimul *40 I.U. PMSG.*

Cholesteri	Changes											
Mg./g.	Pe ce											
−0.3	−	26.8 2.5	−24.3	−90.7	21.6 27.2	+5.6	+25.9	71.5 56.8	−14.7	−20.6	39.2 37.0	− 2.2	− 5.6
−0.2	−	38.0 16.4	−21.6	−56.8	25.0 24.6	−0.4	− 1.6	68.8 53.5	−15.3	−22.2	26.8 26.2	− 0.6	− 2.2
−0.3	−	41.0 10.6	−30.4	−74.1	23.3 31.0	+7.7	+33.0	68.8 52.9	−15.9	−23.1	25.9 25.2	− 0.7	− 2.7
—	—	— —	—	—	— —	—	—	— —	—	—	— —	—	—
−0.6	−21	32.0 4.7	−27.3	−85.3	25.2 —	—	—	89.9 54.5	−35.4	−39.4	51.8 —	—	—
−0.4	−15	44.6 6.8	−27.8	−62.3	23.4 27.7	+4.3	+18.3	97.4 82.3	−15.1	−15.5	52.1 52.6	+ 0.5	+ 1.0
−0.1	+ 3	43.9 11.1	−32.8	−74.7	25.3 31.3	+6.0	+23.7	118.3 68.1	−50.2	−42.4	72.2 39.8	−32.4	−44.9
−1.9	−4	36.3 10.4	−25.9	−71.3	24.7 23.4	−1.3	− 5.3	98.0 67.7	−30.3	−30.9	57.3 45.2	−12.1	−21.1
−0.3	− 1	20.7 20.0	− 0.7	− 3.4	31.0 28.1	−2.9	− 9.4	84.2 88.0	+ 3.3	+ 4.5	49.7 55.9	+ 6.2	+12.5
−0.5	−10		−23.9	−64.8		+2.7	+12.1		−21.6	−23.7		− 5.9	− 9.0
±0.2	± 1		± 3.5	± 9.6		±1.6	± 6.5		± 5.8	± 5.2		± 4.9	± 7.1
±0.6	±13		±10.0	±27.1		±4.2	±17.1		±16.4	±14.8		±12.9	±18.7
8			8			7			8			7	

TA

Animals stimul *h 80*

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholester			E Ch
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Change		g./g.
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	P ce	
25	R 345 L 488	+143	+41.4	49.5 12.8	−36.7	−74.1	3.8 3.3	−0.5	−1	45.7 9.5
30	R 208 L 365	+157	+75.5	34.5 3.7	−30.8	−89.3	3.0 −	−	−	31.5 −
37	L 353 R 443	+ 90	+25.5	49.3 9.5	−39.8	−80.7	4.0 1.6	−2.4	−6	45.3 7.9
43	L 290 R 390	+100	+34.5	42.7 8.1	−34.6	−81.0	3.5 1.9	−1.6	−4	39.2 6.2
49	L 311 R 325	+ 14	+ 4.5	45.5 6.4	−39.1	−85.9	3.6 2.2	−1.4	−3	41.9 4.2
54	L 454 R 529	+ 75	+16.5	17.9 5.0	−12.9	−72.1	2.5 1.7	−0.8	−3	15.4 3.3
57	R 348 L 398	+ 50	+14.4	22.9 7.3	−15.6	−68.1	2.6 2.4	−0.2	−	20.5 4.9
	M.=	+89.9	+30.3		−29.9	−78.7		−1.2	−3	
	S.E.=	±18.9	± 8.9		± 4.2	± 3.0		±0.3	±	
	S.D.=	±50.0	±23.5		±11.1	± 7.9		± 0.8	±1	
	n=	7			7			6		

with the exception of the graded gonadotrophic stimulus. The material comprises 7 groups of experimental animals (Table I—VII). Only pedigreed female white Angora rabbits were used, 67 animals althogether. The mean animal weight was 3.05 Kg., range 2.5 to 3.6 Kg. The animals were kept on a common diet, containing hay, corn, and turnips.

In order to secure as far as possible a constant and welldefined

TA
uls stimul *h 80 I.U. PMSG.*

Esterified Cholesterol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
	Changes			Changes			Changes			Changes	
Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
45.7 9.5	−36.2	−79.2	26.8 32.5	+5.7	+21.3	87.0 61.6	−25.4	−29.2	38.7 33.6	− 5.1	− 13.2
31.5 −	−	−	30.7 −	−	−	62.5 76.9	+14.4	+23.0	21.0 −	−	−
45.3 7.9	−37.4	−82.6	25.2 32.7	+7.5	+29.8	102.8 69.0	−33.8	−32.9	55.8 41.9	−13.9	−24.9
39.2 6.2	−33.0	−84.2	23.2 29.9	+6.7	+28.9	100.0 71.4	−28.6	−28.6	58.4 47.3	−11.1	−19.0
41.9 4.2	−37.7	−90.0	24.7 32.2	+7.5	+30.4	84.3 59.0	−25.3	−30.0	39.9 34.7	− 5.2	−13.0
15.4 3.3	−12.1	−78.6	27.9 31.0	+3.1	+11.1	69.9 55.0	−14.9	−21.3	41.0 32.1	− 8.9	−21.7
20.5 4.9	−15.6	−76.1	31.0 35.4	+4.4	+14.2	93.5 74.2	−19.3	−20.6	59.2 47.3	−11.9	−20.1
	−28.7	−81.8		+5.8	+22.6		−19.0	−19.9		− 9.4	−18.7
	± 4.8	± 2.0		±0.7	− 3.2		± 6.0	± 6.8		± 1.4	± 1.9
	±11.7	± 5.0		±1.8	± 8.5		±15.9	±18.1		± 3.5	± 4.6
	6			6			7			6	

endocrine state with relative inactivity (maximal store of esterified cholesterol), pseudopregnancy was elicited by an intravenous injection of 300 I. U. human chorionic gonadotrophin.[1] The animals were used for experiments on the 12th day of pseudopregnancy.

[1] A.B. Pharmacia, Upsala, has kindly assisted the investigation by placing their preparation PREGNYL at our disposal.

TABLE I

Animals stimulated with 160

Animal No.	Weight of Ovaries			Total Cholesterol			Free Cholesterol			Es Cho
	Mg.	Changes		Mg./g.	Changes		Mg./g.	Changes		g./g.
		Mg.	Per cent		Mg./g.	Per cent		Mg./g.	Per cent	
31	R 297 L 453	+156	+52.5	48.4 9.5	−38.9	−80.4	3.5 2.9	−0.6	−17	44.9 6.6
38	L 298 R 395	+ 97	+32.6	37.6 6.7	−30.9	−82.2	3.7 2.7	−1.0	−2	33.9 4.0
47	R 250 L 376	+126	+50.4	34.3 7.3	27.0	−78.7	3.9 2.9	−1.0	−2	30.4 4.4
48	R 228 L 267	+ 39	+17.1	45.9 8.2	−37.7	−82.1	3.7 3.3	−0.4	−10	42.2 4.9
53	L 285 R 324	+ 39	+13.7	29.7 14.0	−15.7	−52.9	3.5 −	−	−	35.2 −
56	R 415 L 588	+173	+41.7	24.5 5.1	−19.4	−79.2	3.3 1.4	−1.9	−57	31.2 3.7
58	R 274 L 296	+ 22	+ 8.0	22.9 3.4	−19.5	−85.2	2.9 1.9	−1.0	−34	20.0 1.5
	Mean=	+93.7	+30.9		−27.0	−77.2		−1.0	−2	
	S.E.=	±22.8	± 6.8		± 3.5	± 4.2		±0.2	± 6	
	S.D.=	±60.5	±18.1		± 9.3	±11.1		±0.5	±16	
	n=	7			7			6		

As in earlier investigations, the lipid changes were established in the first place by the comparison of the two ovaries of the same animal. The first ovary was extirpated in sodium N-methyl-cyclohexenylmethylbarbiturate[2] anaesthesia immediately before the intravenous administration of gonadotrophic hormone and

[2] A.B. Leo, Helsingborg, has put these preparations at our disposal through the kind assistance of Mr. S. Wiström.

TABLE I

s stimulated with 160 I.U. PMSG.

Esterified Cholesterol			Phospholipids			Total Fatty Acids			Residual Fatty Acids		
	Changes			Changes			Changes			Changes	
Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent	Mg./g.	Mg./g.	Per cent
44.9			25.6			86.1			39.1		
6.6	−38.3	−85.3	34.3	+ 8.7	+34.0	60.0	−26.1	−30.3	−32.7	− 6.4	− 16.4
33.9			25.5			108.4			68.8		
4.0	−29.9	−88.2	25.9	+ 0.4	+ 1.6	62.4	−46.0	−42.4	42.5	−26.3	− 38.2
30.4			24.3			120.0			83.5		
4.4	−26.0	−85.2	31.6	+ 7.3	+30.1	72.5	−47.5	−39.6	48.5	−35.0	− 41.9
42.2			—			104.4			—		
4.9	−37.3	−88.4	29.7	—	—	65.8	−38.6	−37.0	42.7	—	—
26.2			—			92.1			—		
-	—	—	—	—	—	77.7	−14.4	−15.6	—	—	—
21.2			27.8			102.4			69.7		
3.7	−17.5	−82.5	38.7	+10.9	+39.2	72.3	−30.1	−29.4	44.0	−25.7	− 36.9
20.0			28.2			73.1			41.0		
1.5	−18.5	−92.5	29.2	+ 1.0	+ 3.5	59.4	−13.7	−18.7	38.9	− 2.1	− 5.1
	−27.9	−87.0		+ 5.7	+21.7		−30.9	−30.4		−19.1	−27.7
	± 3.6	± 1.5		± 2.1	± 7.9		± 5.3	± 3.9		± 6.3	± 7.2
	± 8.9	± 3.7		± 4.7	±17.8		±13.9	±10.3		±14.1	±16,1
	6			5			7			5	

physiological saline, respectively. The other ovary was removed 12 hours later. Our first aim being to establish the quantitative relationship between the dose of gonadotrophic hormone and the degree of reduction in ovarian cholesterol esters, the interval of 12 hours was chosen on the basis of results previously obtained by us (CLAESSON, HILLARP and HÖGBERG, 1953), which showed that the esterified cholesterol had reached its asymptotic

level after 12 hours' supramaximal stimulation. A longer interval would naturally be possible but might involve a reversion of the processes at the administration of submaximal doses.

The gonadotrophic stimulation was produced by the intravenous injection of pregnant mare serum gonadotrophin — PMSG.[2] The stimulated animals were divided into six groups which received 5, 10, 20, 40, 80 and 160 I. U. PMSG, respectively. Every time operations were performed, fresh solutions of a commercial preparation, Antex Leo, from the same batch were prepared in such a way that the doses administered were always given in a solution of 2 ml. 0.9 per cent NaCl. The control animals received 2 ml. 0.9 per cent NaCl intravenously but were in other respects treated in the same way as the stimulated animals. The distribution of the material on the various groups appears from Tables I—VII.

The lipid determinations were carried out as described in previous papers (Claesson, Diczfalusy, Hillarp and Högberg, 1948; Claesson, Hillarp and Högberg, 1953).

Results

The lipid content of the stimulated ovary was calculated in two different ways in our previous investigations: with consideration to the increase in ovarian weight resulting from the gonadotrophic stimulation (1948, 1953) and without such consideration. In the investigation of the time-relation between the changes in the lipid fractions in the ovary (1953), the lipid content of the ovary last removed was calculated on the basis of its own weight after one and a half and three hours of stimulation. After six and 12 hours' stimulation, however, when the ovary showed a marked increase in weight, the lipid content of the stimulated ovary was calculated on the weight of the ovary first removed (for details see Claesson, Diczfalusy, Hillarp and Högberg, 1948). This mode of calculation was considered justified, as experiments with colchicine treated animals had shown that the increased weight of the ovary following gonadotrophic stimulation was not due to an increase in the number of cells (Claesson, 1954 b). Naturally, this experiment does not exclude

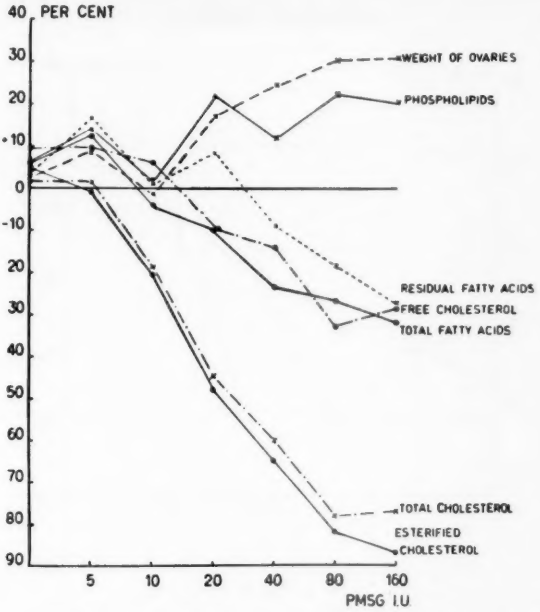

Fig. 1. Changes of the lipids in the ovarian interstitial gland and of the ovarian weight at gonadotrophic stimulation (pseudopregnant rabbits). The first ovary extirpated immediately before the intravenous administration of 5—160 I.U. PMSG, the other ovary removed 12 hours after the injection.

the possibility of an increase in the cytoplasmic structures of the individual cells, which, partly in addition to hyperaemia and tissue fluid accumulation, may thus cause an increase in the ovarian wet weight.

In the present investigation, where the experimental time is fixed at 12 hours and the dose of gonadotrophic hormone is the variable, calculations must be carried out in such a way that the interpretation of the results becomes independent of possibly occurring cytoplasmic increase, hyperaemia and tissue fluid accumulation during the time of stimulation. Hence in every animal in all the groups the weight of the ovary first removed was made the basis of the calculation of the lipid con-

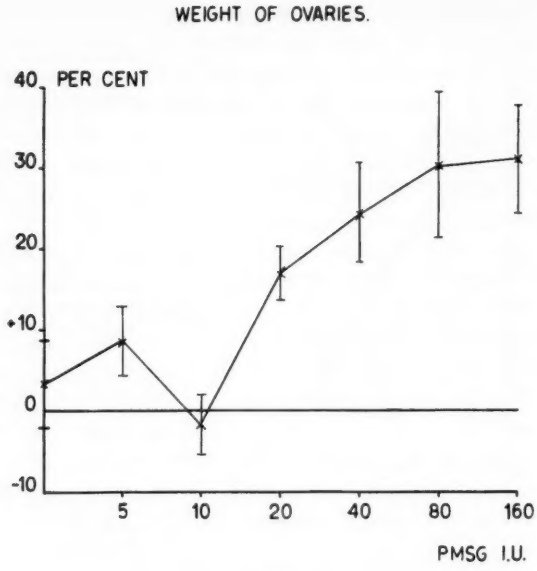

Fig. 2.

tent in the second ovary. As regards the lipid fractions which undergo a reduction at stimulation with gonadotrophic hormone, this way of calculating offers the only means of establishing *the actual reduction in the stimulated cell*, independent of extracellular factors as, for instance, hyperaemia. Even though these factors might be neglected or determined, this systematic error would be introduced when an increase in the amount of cytoplasm of the interstitial gland occurs, a possibility which cannot be excluded, as was pointed out above. The calculation of the phospholipid changes will be discussed below.

The mode of calculation in the present paper is based on the assumption that the two ovaries of one and the same experimental animal are so similar in weight at the beginning of the experiment that the initial negative and positive differences between all the ovaries within an experimental group are practically compensated. The correctness of this assumption is evident

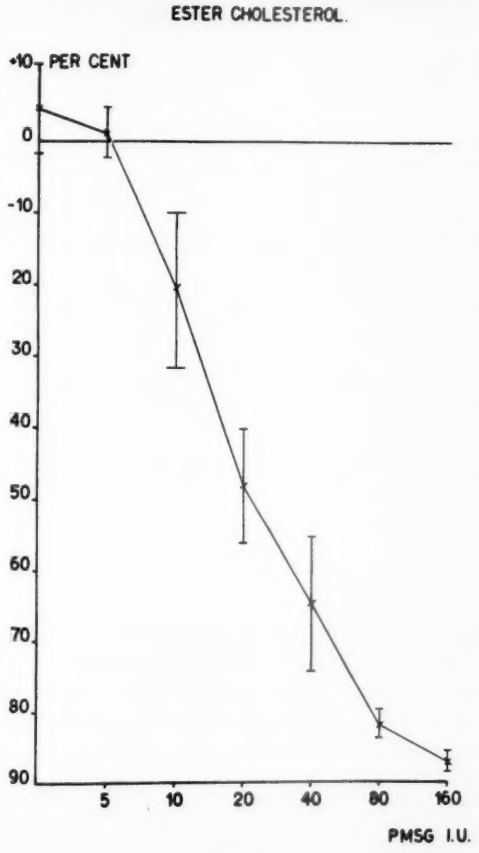

Fig. 3.

both from previous investigations (Claesson, Diczfalusy, Hillarp and Högberg, 1948) and from the experimental group of control animals presented in this study (Table I).

The results of the determinations comprising the ovarian weight and the various lipid fractions of every single animal in all the experimental groups are given in Tables I—VII. The lipid content is expressed in mg./g. of ovarian tissue in accor-

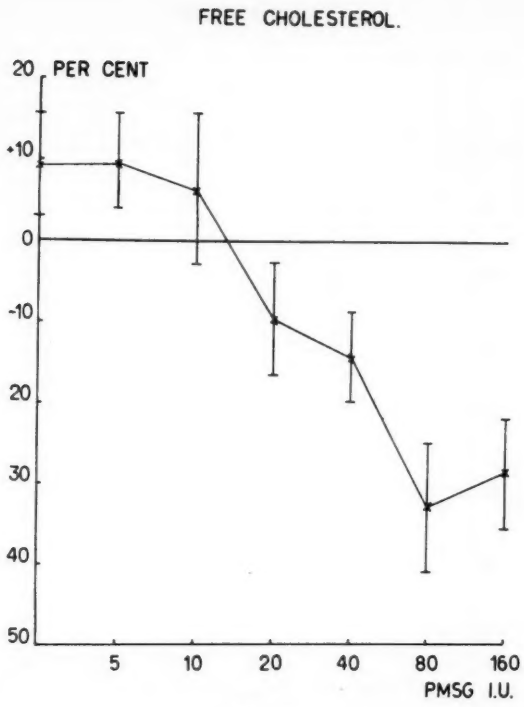

Fig. 4.

dance with the mode of calculation described above. The lipid changes in the ovaries removed after 12 hours' stimulation are given both in mg./g. and in per cent. The changes of the lipids and of the ovarian weight, expressed in per cent, are represented graphically in relation to the dose of PMSG in Fig. 1; Figs. 2—7 show the course of every particular component with a vertical line through each point representing the standard error of the mean. If the changes are expressed in mg./g., they show the same course in relation to dose of gonadotrophic hormone as do the curves presented in Figs. 1—7.

As appears from Table I and Figs. 2—7, the control animal group does not show any certain changes either in ovarian

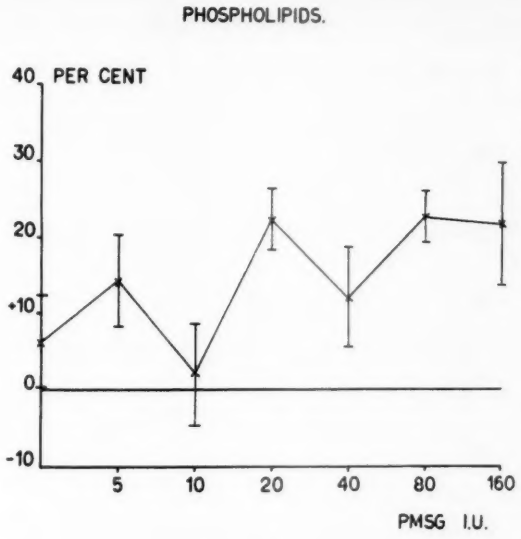

Fig. 5.

weight or in the determined lipids. Under the experimental conditions used neither operation trauma nor the removal of one ovary produce any changes within 12 hours in the weight and in the lipid content of the remaining ovary. It is thus clear that the experimental conditions used make an investigation of the quantitative relationship between dose of injected PMSG and degree of lipid changes possible.

The chlosterol esters are naturally of primary interest in the present paper. As can be seen from Figs. 1, 3 and 9, this fraction undergoes a continuous and marked reduction which, to judge from its course in relation to dose of PMSG, is likely to have started already below 10 I. U. and almost reached its lowest level at 80 to 160 I. U. (mean of reduction 81.8 ± 2.0 and 87.0 ± 1.5 per cent or 28.7 ± 4.8 and 27.9 ± 3.6 mg./g., respectively).

The degree of reduction in esterified cholesterol seems actually to be almost directly proportional to log dose of injected PMSG.

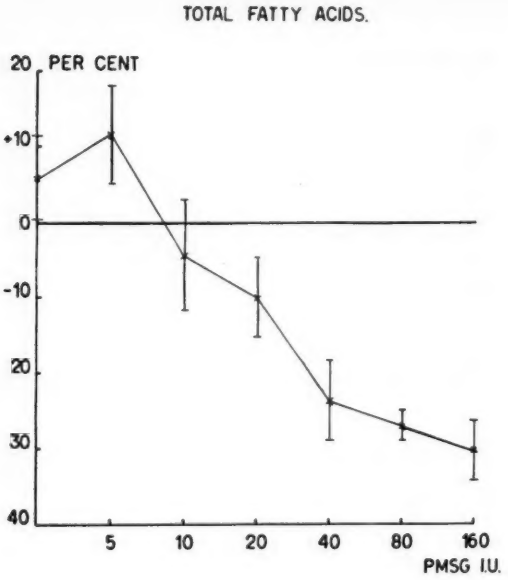

Fig. 6.

With regard to the reduction of the esterified cholesterol in relation to other ovarian changes observed, it is evident from Fig. 1 that this fraction undergoes a distinct reduction ($M = -20.7 \pm 10.8$ per cent or -6.8 ± 3.8 mg./g.) on stimulation with 10 I. U., a dose that does not cause any other demonstrable changes in the ovary.

The non-esterified cholesterol shows a clear reduction, probably beginning at doses smaller than 40 I. U. At a dose of 80 to 160 I. U. this reduction amounts to about 30 per cent (1.2—1.0 mg./g.).

The total fatty acids (Figs. 1, 6 and 8) show a reduction which at 160 I. U. is as much as 30.4 ± 3.9 per cent. The amounts of fatty acids disappearing from the cells is considerable, 30.9 ± 5.3 mg./g. of ovarian tissue. The course of this decrease indicates

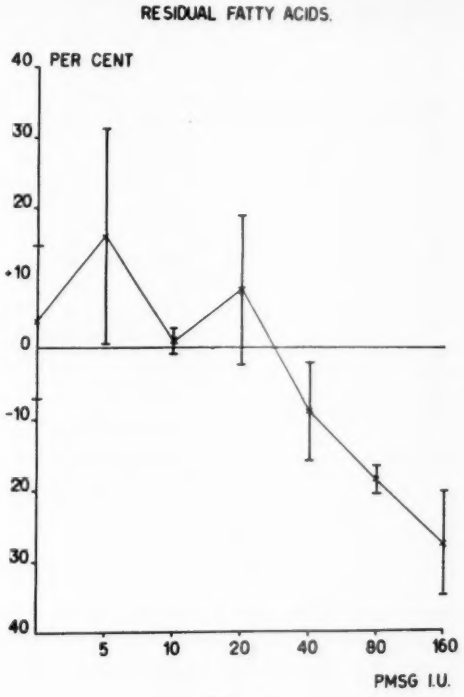

Fig. 7.

a starting-point at a dose of 20 I. U. PMSG ($M = 10.0 \pm 5.2$ per cent or 7.9 ± 3.7 mg./g.).

The calculated fraction of residual fatty acids is difficult to evaluate (Figs. 1, 7 and 8). There is, however, an unmistakable reduction at 80 and 160 I.U., for the latter dose amounting to 27.7 ± 7.2 per cent or 19.1 ± 6.3 mg./g. The present material does not permit an establishment of the changes in this fraction at lower doses.

The weight of the ovary first removed was made the basis of the calculation also concerning the phospholipids which show an increase at stimulation. It is obvious that such a mode of calculation could not give any information of real value as re-

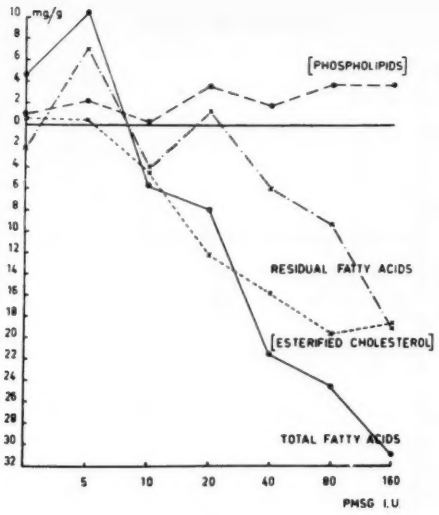

Fig. 8.

gards the stimulated gland cell if the increased weight of the stimulated ovaries were in any degree due to an increase in the number of cells. The increase in phospholipids and in ovarian weight occurring during the first 12 hours of gonadotrophic stimulation cannot, however, be caused by this factor to any larger extent (for details, see CLAESSON 1954 b). From this point of view the method of calculation used is adequate. This method, therefore, was chosen on account of the errors of unknown magnitude (increase in weight through hyperaemia, etc.) which are introduced if the opposite procedure is used, i. e. if the calculation is based on the stimulated ovary's own weight.

As appears from Figs. 1 and 5, there is an increase in phospholipids which seems to take place at the same dose level as the increase in ovarian weight and amounts to 22.2 ± 4.1 per cent or 5.4 ± 1.2 mg./g. at 20 I.U. No further increase is evident at higher dose levels, while the ovarian weight, on the other hand, shows a continuous rise (20 I.U. 17.0 ± 3.3; 160 I.U. 30.9 ± 6.8 per cent. Figs. 1 and 2).

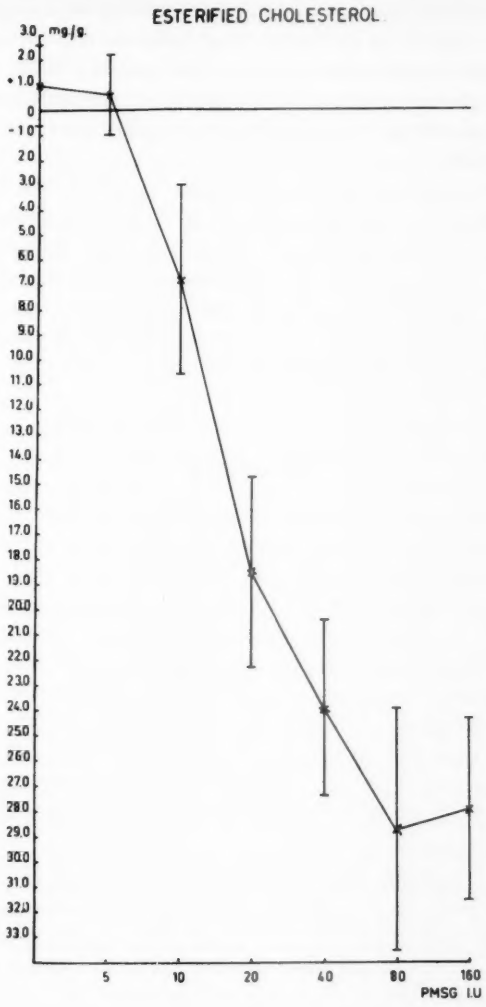
ESTERIFIED CHOLESTEROL
mg/g
30
20
+ 1.0
0
- 1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.0
32.0
33.0
5
10
20
40
80
160
PMSG I.U.

Fig. 9.

The observed increase in phospholipids is of such a magnitude that even if the entire maximal increase in ovarian weight was due only to an increased blood content, the blood phospholipids would nevertheless contribute with one tenth of the observed values at the most (cf. CLAESSON, DICZFALUSY, HILLARP and HÖGBERG, 1948).

In the experimental group which received 5 I.U. PMSG (Table II), all the fractions except the cholesterol esters showed a tendency to increase. It is not entirely out of question that this may be an effect of the gonadotrophic stimulation. This is not very likely, however, since the mean values are close together in the same way as in the control animal group and in the experimental group 10 I.U. (Fig. 1).

Discussion

In previous studies on the cholesterol esters in the interstitial gland of the ovary, their changes in various functional states of the gland cells were investigated (CLAESSON, HILLARP et al. 1946—1949, 1954; CLAESSON, 1954). The results of these investigations have step by step corroborated our assumption that the esterified cholesterol stored in the interstitial cell is a precursor of oestrogenic hormones.[1] A quantitative study (1953) dealing with the reduction of this cholesterol fraction at supramaximal gonadotrophic stimulation and its course in time, furnished the basic facts necessary for a crucial test of our assumption, viz. an analysis of the quantitative relationship between the dose of

[1] LEVIN and JAILER (1948) suggest that the interstitial gland does not produce oestrogenic hormone but rather progesterone. They do not, however, discuss the numerous investigations which show that the progesterone of the ovary is formed in the system of granulosa cells and corpora lutea and only there. Nor do they mention the investigations which show, almost beyond doubt, that the oestrogen secretion should be referred to the follicular wall, interstitial gland, and corpora lutea containing theca interna cells. Nor do they discuss the wellknown experiment on the effect of chorionic gonadotrophin on the ovary of hypophysectomized rat. Ever since the publication of the first studies of COLLIP, SELYE and THOMSON (1933), it has been known that in such an ovary, with atrophic follicles and without corpora lutea, this hormone produces an abundant oestrogen secretion and cellular changes in the interstitial gland and the theca interna only.

injected gonadotrophic hormone and the degree of reduction in the esterified cholesterol.

With regard to the esterified cholesterol, the present investigation demonstrates some characteristics essential for the interpretation of the function of this cholesterol fraction in the gland cell. Practically all the decrease of this cholesterol fraction takes place within the narrow range of doses from 5 to 40 I.U. (Fig. 9). The reduction occurring within this range is very pronounced, as much as 24.5 mg./g. of ovarian tissue, i. e. 83.6 per cent of the maximal reduction at a dose level of 80 to 160 I.U. (29.3 and 28.5 mg./g., respectively). The importance of these values can be clearly understood from the fact that the large amounts of esterified cholesterol stored in the gland cell (about 4 per cent of the wet weight) are almost depleted during the stimulation. Another characteristic is the reaction of this cholesterol fraction to a dose of 10 I.U. (a decrease amounting to 25.3 per cent of the maximal reduction), a dose level at which neither the other lipids determined nor the ovarian weight show any demonstrable changes. Thus, just as the esterified cholesterol is the earliest reacting fraction at gonadotrophic stimulation (-15.2 ± 4.0 per cent or -6.1 ± 1.6 mg./g. at $1\,{}^{1}/_{2}$ hours, CLAESSON, HILLARP and HÖGBERG, 1953), it is also found to react at a lower dose level of PMSG than do the other lipids or the ovarian weight.

In conclusion, the observed course of the reduction in the esterified cholesterol stored in the ovarian interstitial gland in relation to dose of PMSG is on no point inconsistent with the assumption of this esterified cholesterol being a precursor of oestrogenic hormones. What is more, the entire course of the reduction is such as might be expected from a stored precursor under the experimental conditions given. A general discussion of the esterified cholesterol as a precursor of oestrogenic hormones will be presented in another paper (CLAESSON, 1954 d).

The total fatty acids in the interstitial gland cells undergo a decrease, which at increasing doses up to 40 I.U. largely seems to reflect a dissappearance of the fatty acids in the esterified cholesterol fraction (Fig. 8). At higher dose levels, where the cholesterol esters have reached their lowest values, the total fatty acids, however, show a continued decrease. At 160 I.U.

the decrease is considerable, 30.9 ± 5.3 mg./g. Since the fatty acids in the phospholipids constitute only a small portion of the total fatty acids and their increase, expressed in mg./g., is comparatively small also at the higher doses, the fraction »residual fatty acids» must consequently decrease after the maximal reduction of the cholesterol esters. As appears from Fig. 8, only a slight fraction of the fatty acids of the esterified cholesterol can conceivably enter into the new-formed phospholipids. The part played in the specific function of the cell by the fatty acids disappearing from the determined fraction »total fatty acids» cannot be established at present. The considerable reduction which takes place in these fatty acids seems, however, to indicate that they are linked up with some essential process caused by the gonadotrophic stimulation.

Gonadotrophic stimulation causes an increase in the phospholipids, amounting to about 22 per cent. This change in the phospholipids coincides with the increase in ovarian weight both as regards dose and time (Claesson, Hillarp and Högberg, 1953). The significance of this fact cannot be evaluated at present. The increase is most likely due to a formation of phospholipids within the stimulated gland cell. The significance of this formation is naturally difficult to interpret as there is no means of deciding in which cell component the formation takes place. It has been demonstrated, however, through separation of the different cell components, that the increase in phospholipids probably is an expression of a formation of cytoplasmic particles, probably of mitochondrial nature (Claesson, 1954 d). In such a case the increase in phospholipids observed in the present investigation would indicate that this formation is generated even by relatively small doses of PMSG.

Summary

The lipid changes in the interstitial gland of the pseudopregnant rabbit ovary have been investigated 12 hours after the administration of 5 to 160 I.U. pregnant mare serum gonadotrophin.

The observed course of the reduction in the esterified chol-

sterol in relation to dose of PMSG is in no respect inconsistent with the assumption that this cholesterol fraction is a precursor of oestrogenic hormones. What is more, the entire course of this reduction is such as might be expected from a stored precursor under the given experiment conditions.

The phospholipids show an increase and the fatty acids a considerable decrease at increasing gonadotrophic stimulation. These changes are discussed in relation to the reduction in cholesterol esters and the increase in ovarian weight.

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From the Department of Histology, Lund, Sweden

The Intracellular Localization of the Esterified Cholesterol in the Living Interstitial Gland Cell of the Rabbit Ovary[1]

By

LENNART CLAESSON

All the cells producing steroid hormones are characterized by a high storage of esterified cholesterol under certain functional stages. Considering the important rôle of this cholesterol fraction at the biosynthesis of steroids it is essential to know the intracellular localization and structural organization of the stored cholesterol in the living gland cell. Investigations on this problem are practically lacking.

This problem has been taken up as a necessary link in previous investigations on the cholesterol of the interstitial gland in the ovary. The cytoplasmic components of the living interstitial cell in the rabbit ovary have been separated and examined with special regard to the stored cholesterol and the cytological changes at gonadotrophic stimulation.

Material and Methods

Only pedigreed female white Angora rabbits on the 12th day of pseudopregnancy were used, as in previous works (CLAESSON, 1954 c).

The experiments were performed at two different functional stages of the interstitial gland cell, stage of cholesterol storage and stage of cholesterol mobilization at gonadotrophic stimulation.

[1] The investigation has been aided by a grant from the Faculty of Medicine, Lund.

1. Examination of the interstitial cells after fixation. In 18 animals one ovary was removed in sodium N-methyl-cyclohexenylmethylbarbiturate[1] anaestesia immediately before the intravenous injection of 450 I.U. pregnant mare serum gonadotrophin — PMSG (Antex Leo[1]), and the other ovary after the elapse of 3, 6, 12 and 24 hours respectively. Each ovary was divided crosswise into two parts. The one half was fixed at +4° C. during 3 days in 10 per cent formalin containing one per cent $CaCl_2$ according to BAKER (1946), and 10μ frozen sections were stained with Sudan black B: Sections were washed for a few seconds in 50 % alcohol, then stained for 10 minutes in Sudan black B (0.1 g. in 100 ml. 70 % alcohol). They were then washed twice for about 10 seconds in 50 % alcohol and mounted in glycerine. The Schultz reaction was applied to other frozen sections (CLAESSON, 1954 a). The second half was fixed 12 hours in Susa according to Heidenhain or in Bouin's solution (15 volumes of a saturated aqueous solution of picric acid, 5 volumes of 40 per cent formalin, 1 volume of glacial acetic acid) and embedded in paraffin via methylbenzoate and benzene. Sections (8μ) were stained in unbuffered gallocyanin-chrome alum according to Einarson and in haematoxylin-eosin.

2. Examination of the living cell. Small cell aggregates were dissected from the interstitial gland with fine needles (before as well as after 3—12 hours of gonadotrophic stimulation) and were immediately transferred to 0.15 M NaCl, KCl of 0.3 M sucrose on the microscopic stage at +38° C. The cells were examined by light-field and dark-field illumination and with polarized light. A Leitz polarizing microscope (oil-immersion objective, 100×, n. A. 1.3) was used with a light-source of high intensity (high-pressure mercury lamp). The character and magnitude of the birefringence were determined with a compensator according to Berek.

3. Separation of the cytoplasmic structures. The ovaries were removed immediately after killing the animals and cooled by ice. The corpora lutea were removed, the larger Graafian follicles punctured and the ovaries were weighed on a torsion balance.

[1] Mr S. Wiström, A.-B. Leo, Helsingborg has kindly put these preparations at my disposal.

The ovaries were pressed through a cold plexiglass sieve with holes having a diameter of approx. 0.5 mm. The pulp thus obtained was suspended in ice-cooled 0.3 (or 0.8 M) sucrose solutions, which have been shown to be the most suitable ones for isolation purposes (HOGEBOOM, SCHNEIDER and PALADE, 1948, HOGEBOOM, 1951, SCHNEIDER and HOGEBOOM, 1951). The suspension was homogenized according to POTTER and ELVEHJELM, using a pestle of plastics rotating in a test tube at about 300 r.p.m. The pestle diameter was about 0.2 mm. less than the tube diameter (cf. WILBUR and ANDERSON, 1951). In homogenizing, the pestle was moved ten times up and down.

Unbroken cells, connective tissue and nuclei were removed from the ice-cooled homogenate (0.3 M sucrose) by means of centrifugation at $800 \times g$ for 2 minutes (Wifug angle head centrifuge, type BM. The centrifugal forces are calculated for the bottom of the tubes). After the centrifugation the lipid granules floating on the surface were carefully suspended in the supernatant which thereafter was transferred to a new centrifuge tube kept on ice. The residue was washed once by resuspending in 3 ml. 0.3 M sucrose and recentrifuging for 3 minutes at the same force. The two supernatants were combined and recentrifuged at $800 \times g$ for 3 minutes. The resulting supernatant was transferred to a test tube on ice, after resuspending the floating lipid granules. Dark-field microscopical examination showed this supernatant (whole cytoplasmic fraction) to be free from cells and nuclei.

The whole cytoplasmic fraction was separated into three subfractions by centrifugation at $6.800 \times g$ for 30 minutes (Wifug angle head centrifuge, type R, operating at $+4°$ to $+9°$ C.): a) Lipid granules floating on the surface, b) Large granules firmly packed on the bottom, c) Remaining cytoplasmic fraction (ground cytoplasm and small granules, see Results).

For the quantitative determinations of the lipid distribution in the three isolated cytoplasmic fractions the method was as follows:

The ovaries from 8 animals were homogenized in 10.0 ml. 0.3 M sucrose and the homogenate was centrifuged at $800 \times g$ as above. From the "whole cytoplasmic fraction" obtained 2.0 ml. were

removed for lipid determinations (see below). A measured volume (9—10 ml.) from the remaining part of this fraction was transferred to four centrifuge tubes and separated in three subfractions as above.

The lipid granules, together with a small amount of the supernatant, were withdrawn to a test-tube by means of a capillary pipette. The remaining part of the supernatant was withdrawn to another test tube, and 5.0 ml. of this "remaining cytoplasmic fraction" were used for lipid determinations.

Remaining parts of the lipid granule layer adhered to the walls of the centrifuge tubes were thereafter removed by carefully wiping with small pieces of filter paper. After removing the bottom fraction, "large granules", the centrifuge tubes were washed twice with ethanol-ether to dissolve the last traces of lipid granules. The ethanol-ether from these washings, the used pieces of filter paper and the lipid granules withdrawn at the first step were combined for lipid determinations. — The volume of the lipid granules withdrawn at the first step containing a contaminating amount of the supernatant, was measured. Since the lipid granules amounted only to a small part (one tenth to one twentieth) of the volume measured, this volume was regarded as constituting the contaminating supernatant volume at the calculation of the lipids in fraction "lipid granules".

The removal of the large granule fraction in each centrifuge tube was performed by suspending in 2×0.3 ml. water and withdrawing by means of a capillary pipette.

The four different fractions of the ovarian homogenate were extracted three times with a 3:1 mixture of ethanol and peroxide-free ether, boiling for 5 minutes. After filtering the extracts were made to a volume of 100.0 ml. For all of the lipid analyses various aliquots of these extracts were used.

The lipid determinations were performed in the same way as in previous works (Claesson, Diczfalusy, Hillarp and Högberg, 1948).

Results

1. *The cytology of the interstitial cell after fixation.*

The interstitial gland of 18 animals was examined histologically. In order to increase the reliability of the observations of the actual cytoplasmic changes at gonadotrophic stimulation, the two ovaries of the same animal were compared throughout the investigation, on account of which one ovary was removed immediately before this stimulation.

Sudan black B: All the unstimulated ovaries presented the same picture. The cytoplasm of the gland cells was loaded with dark blue granules of variable size, from very fine ($<0.5\mu$) to relatively large granules (2—3μ); but on the whole the granules were relatively uniform in size, approx. 1—1.5μ. Moreover, there was found a quite different type of granules, colourless or faintly sudanophilic and of very small size. They seemed in the main to be centrally placed round the nucleus and existed in great numbers. On the whole the interstitial cells were of the same appearance throughout the gland and had the same large number of the intensely Sudan coloured granules. In some small cell groups, however, the cells were rich in larger globules, intensely sudanophilic and varying in size. — In the whole gland, inside and partly outside the cells, there was an abundance of colourless, needleshaped crystals. These crystals were soluble in fat solvents and showed a strong birefringence, hence probably being crystallized lipids.

The stimulated ovaries were removed 3, 6, 12 and 24 hours respectively after the intravenous injection of 450 I.U. PMSG. This dose gives a supramaximal stimulation as regards the reduction in esterified cholesterol (Claesson, Hillarp and Högberg, 1953, Claesson, 1954 c.).

These ovaries did not show any marked changes in the interstitial gland cells at comparison with the unstimulated ovaries. The only significant difference was the fact that the needle crystals could no longer be seen. Concerning the Sudan coloured granules the larger granulations seemed to be reduced in number; but there was still an abundance of small granules,

intensely sudanophilic. The smaller, colourless or faintly coloured granules seemed to be unchanged.

Schultz reaction: The interstitial cells in the unstimulated ovaries showed — as previously described (CLAESSON and HILLARP, 1947 a) — an intense reaction. The cells were loaded with blue-green coloured granules of about the same size as the sudanophilic granules. Contrary to this, the interstitial cells gave a considerably reduced reaction already after 3 hours of stimulation. The reaction was almost quite negative in the ovaries stimulated during 6, 12 and 24 hours respectively. There were, however, some small cell groups with coarse globules, varying in size and intensely green coloured by the Schultz reagent. — The needleshaped crystals uniformly distributed throughout the interstitial gland of the unstimulated ovaries, did not show any definite positive reaction.

Gallocyanin-chrome alum. No definite changes were noted in the nuclear structures at gonadotrophic stimulation. Possibly there was a tendency to an increase in the nuclear volume and in the size of the nucleolus, which is small and often indistinct in the unstimulated cells. — The cytoplasm of the interstitial gland cells had — both before and after stimulation — a fine-granular appearance and was only faintly basophilic. After 6—24 hours of stimulation the picture of all cells was suggestive of an increase in the cytoplasm volume. It is necessary, however, to make quantitative measurements of the volumes of the cellular components to prove the correctness of the sesuggestions.

Discussion. Among the numerous methods for a histologic demonstration of the cell lipids the Sudan black technique has proved to be the most sensitive and satisfactory (CAIN, 1950). However, it is impossible to differentiate the various lipids from each other by this method. It seems, at present, not to exist any histochemical method, which has such a specificity and is sufficiently examined as to make a definite differentiation possible (apart from the plasmal[1] and the Schultz reaction, cf. EVERETT, 1947, CLAESSON and HILLARP, 1947 a). Hence no attempts have

[1] The plasmal reaction according to Feulgen has not been used as the acetal phosphatides are of less interest at the present stage of the investigation.

been made to apply such methods at investigation of the ovarian interstitial gland (cf. SAYERS, 1950).

The Sudan black method shows the interstitial gland cells to be loaded with granules of probable lipid nature. These lipid inclusions have been demonstrated in a number of thorough investigations on the adrenal cortex, corpus luteum and the ovarian interstitial gland (e. g. EVERETT, 1947, YOFFEY and BAXTER, 1949, CAIN and HARRISON, 1950, RENNELS, 1951, 1952). However, concerning an investigation of the different lipids in the interstitial gland and their alterations at gonadotrophic stimulation the Sudan black method is highly limited. This is shown by the following reasons: a) The sudanophilic granules and the Schultz positive granules cannot with certainty be considered identical, as Sudan positive granules are still demonstrable in great number at the total depletion of Schultz reactive substances by gonadothrophic stimulation. b) The lipid changes in the interstitial gland at gonadotrophic stimulation, demonstrated by quantitative determinations, are not parallelled with changes demonstrable by means of Sudan black. c) The method does not permit a determination of the constituents of the demonstrable lipid granules and does not even give any possibility of deciding if these granules are vital structures existing in the living cell. That the cell lipids undergo considerable alterations at fixation, is evident from the fact that some lipid constituent crystallizes as needles in the cells. The same artefact has been demonstrated by YOFFEY and BAXTER (1947) in the adrenal cortex.

Concerning the Schultz reaction the comparison between the unstimulated and the stimulated ovaries in the present material supports the conception that this reaction applied to the ovarian interstitial gland gives a good estimation of the larger quantitative changes in the cholesterol content as determined by chemical methods (CLAESSON, HILLARP and HÖGBERG, 1953).

II. *The cytology of the living interstitial gland cell.*

As is evident from section I the intracellular lipids in the interstitial gland cannot for the present be localized with certainty by means of the usual lipid staining procedures. When

applying these methods it is not even possible to decide whether the lipid granules demonstrable in the steroid hormon producing cells are vital structures existing in the living cells.

Observations on isolated cells. Single cells and smaller cell groups were teased out from ovaries before and after gonadotrophic stimulation. The isolated cells were transferred to 0.15 M NaCl, KCl or 0.3M sucrose and examined in the light field, dark field and polarizing microscope. The cells had the same appearance in the three different media.

The unstimulated cells were heavily loaded with granules relatively uniform in size (about 1 μ). The granules were so closely packed that they covered the other cell structures, the nucleus hardly being visible. In case of rupture of the cell membrane the granules flew out into the medium without demonstrable changes in their structure. The granules were highly refractive and in polarized light they showed a strong birefringence and a spherite texture, the character of the double refraction being positive with reference to the radially oriented optical axis. These findings are highly suggestive for a lipid nature of the granules.

At gonadotrophic stimulation of the interstitial cells the lipid granules presented pronounced changes in size and birefringence. Already after 3 hours of stimulation there was a significant decrease in the double refraction, but the size seemed to be unchanged. After further stimulation for 3 hours the granules still showed a distinct birefringence which, however, was very weak and could no longer be measured. No further changes in birefringence could be observed in case of stimulation for 12 and 24 hours respectively. There was, however, a progressive decrease in size of the lipid granules, which, after 24 hours of stimulation had a diameter probably not exceeding 0.5 μ. The granules were found in large numbers in the cells even after this stimulation; on account of their highly reduced volume, however, it was not possible to decide if they had decreased in number or not.

Thus the investigation shows the presence of specific granular structures of probable lipid nature which undergo a pronounced decrease in size and birefringence at gonadotrophic stimulation.

A further analysis of the structure of these granules, however, requires their isolation and this has been performed by means of differential centrifugation.

Observations on isolated lipid granules. The lipid granules were isolated from ovarian homogenates in 0.3 or 0.8 M sucrose. During the course of the experiments no difference could be detected between granules isolated in 0.3 and 0.8 M sucrose respectively or isolated by centrifugation at room temperature and at $+4^\circ$ to $+9^\circ$C. respectively. The granule fraction obtained at the final centrifugation consisted of a well defined, white surface layer of a creamy consistency. There was no difficulty in resuspending the granules in sucrose by means of a capillary pipette without their being agglutinated or damaged. By this separation technique it was possible to wash the granules three times with preservation of the same characteristics as in the living cell.

The lipid nature of the isolated granules was proved by their solubility in ethanol, ethyl ether and chloroform. There was, however, an inconsiderable insoluble residue left.

Irrespective of their size the lipid granules showed a strong birefringence of the same sign. In spite of a thorough examination of a great number of preparations from different homogenates it was not possible to demonstrate any isotropic granules. Already after a few minutes all the granules (washed three times in water or sucrose) became faintly but distinctly brown-coloured when suspended in one per cent OsO_4; no further reduction took place for 24 hours. Lipid granules suspended in neutral red (1 : 10000 sucrose solution) for $^1/_2$ to 24 hours turned yellow; no colourless granules could be detected.

Attempts to differentiate the lipid granules in different types on the basis of their specific gravity, were not too successful. On account of the fact that their specific gravity is lower than that of water, such a separation must be performed in organic solvents. Suspensions of granules in a series of centrifuge tubes containing aqueous ethyl alcohol of increasing concentrations (30—50 per cent v./v.) were centrifuged at $6800 \times g$ for 75 minutes at $+15^\circ$ C. It was not possible to demonstrate any type of granules differing from the other ones as regards the

specific gravity, but since the granules were gradually damaged at increasing alcohol concentrations the result is not quite reliable. — Under the given experimental conditions their specific gravity was somewhat below 0.96.

Thus, the isolated granule fraction exclusively (or at any rate almost exclusively) seems to consist of one type of granules. This is furthermore supported by their uniform changes at gonadotrophic stimulation of the interstitial gland cell, as described above. The strongest support for this conception is, however, the high value of the double refraction which is a characteristic feature of apparently all granules from unstimulated gland cells (see below).

At calculation of the double refraction of the lipid granules, the retardations were measured with a Berek compensator. The diameter of the granules was measured on micro-photographs of the same preparations (oil-immersion objective, Apochrom. 90×, n. A. 1.30). The measurements of the retardation and of the size could, however, not be performed on exactly the same granules; but an estimation of sufficient accuracy for the purposes of this investigation could be obtained by a determination of the average size of granules and by measuring the retardation of a large number of granules, avoiding the small and large variants. The average diameter of granules in 0.3 M sucrose was approx. 1 μ and the average of retardation approx. 24 mμ (Γ max.). The double refraction was calculated from the formula for spherites (FREY-WYSSLING, 1953): $\Delta n = \frac{\Gamma \text{ max.}}{1.122 \text{ r.}}$. Under the given conditions the birefringence was approx. 0.04.

Investigations on the effect of various treatments on the isolated lipid granules showed that they had a striking stability:

No changes in the morphology or the optical properties of granules could be observed when keeping washed granules in 0.3 M sucrose at $+4^\circ$ C. for 48 hours.

Drying of the granules in the air at room temperature gave no obvious decrease in their size which could be microscopically detected. (On account of their high refractive index it was difficult to measure their diameters on microphotographs of these preparations.) Nor did the birefringence seem to undergo

any change. Still more remarkable, however, was the fact that lipid granules suspended in water seemed to be quite normal even when kept for 24 hours at about +20° C. Neither swelling nor any obvious decrease in double refraction could be observed.

Lipid granules suspended in 0.3 M sucrose showed a high stability of their crystalline organization at increased temperature. When measuring the double refraction on a microscopic heating stage the birefringence began to decrease and the black cross of polarization became irregular at +78° to +80° C., as a sign of disorganization of the molecular arrangement. When suspended in glycerine, however, the granules lost their double refraction already at a temperature of +52° to +54° C. — After cooling the granules regained their normal double refraction, indicating a recrystallization.

An investigation on the effect of detergents on the lipid granules showed that neither anionic nor cationic detergents caused any microscopically detectable changes (10^{-2} m cetyl pyridinium bromide and sodium laurylsulfonate). Nor did a saturated solution of digitonin in 0.8 M sucrose have any effect. The lipid granules thus have a pronounced resistance to these substances, wellknown for their high activity in disorganizing biological structures. No effect of urea in protein denaturing concentrations (8 M) could be observed.

The lipid granules suspended in a series of buffer solutions were stable between pH 4.5 and 10.0 (1 M acetic acid acetate buffer, 0.1 M phosphate buffer and $^1/_3$ M ammonia ammonium chloride buffer).

Contrary to the above-mentioned high stability at various treatments the lipid granules disintegrated when kept for 48 hours at about +20° C. in 0.3 M sucrose. The granules aggregated and formed greasy irregular masses. A large number of needleshaped crystals were also formed. These crystals showed a strong birefringence, negative with reference to their length, and had the same appearence as the needles in fixed interstitial gland cells.

Observations on the birefringence of lipids extracted from the granules. The lipid granules from 4 ovaries were isolated in

0.3 M sucrose and washed three times in water. They were then extracted with a 3 : 1 mixture of alcohol-ether at room temperature, and this extract was evaporated *in vacuo* (N_2 atmosphere, outside temperature not above $+50°$ C.). The residue was dissolved in chloroform (in which it was completely soluble). The chloroform was evaporated *in vacuo*, and the residue was left to dry over P_2O_5 *in vacuo* (N_2) for ten days.

The dry lipid residue was colourless, semi-transparent, and greasy. Examined in polarized light it proved to contain numerous fine needle crystals with high birefringence, negative with reference to their length, in an apparently homogenous, isotropic mass, which, however, showed a strong orientation double refraction at smearing (positive with reference to the smearing direction). In emulsions obtained by rubbing the dry residue with water or glycerine a rapid formation of spherites took place. The spherites were of small size (about the same size as the lipid granules) and they possessed a birefringence (positive spherites) which was relatively weak in comparison with that of the lipid granules. On the other hand, if the dry lipid mixture was dissolved in chloroform, negative spherites were formed at evaporation of the solvent; they showed, however, a reversal of their optical character at further evaporation of the chloroform.

Discussion. In the living interstitial gland cell of the pseudopregnant rabbit ovary there are stored high amounts of lipids in specific structures, the lipid granules. Gonadotrophic stimulation (PMSG) rapidly produces characteristic changes in these granules with regard to their size and optical properties.

Evidently the isolated granules consist mainly of lipids, most of which are esterified cholosterol (see section III, Table I). It has not been possible to produce any evidence of a presence of a protein component within the granules. But it is reasonable to suggest that the granules have a surface protein membrane. The difficulty in making the granules coalesce indicates the presence of such a membrane. On the whole it is difficult to imagine the existence of granules, consisting of lipids, suspended in the living cytoplasm without a covering protein layer, already on account of surface tension forces (cf. Danielli, 1938, Davson

and DANIELLI, 1943). The fact that detergents and 8 M urea does not give any disintegration of the granules suggests, however, that this presumed membrane either exists as a denatured protein layer or is of no importance to the stability and structure of granules.

The most prominent feature of the lipid granules is the fact that they have a spherite texture with radially oriented optical axis, the character of the birefringence being positive with reference to this axis. This shows that the molecules of the lipid constituents must have a fixed submicroscopic organization. On the basis of the most reasonable assumption viz. that the lipid molecules giving this structure here as in general, are anisodiametric molecules with double refraction of positive character with reference to their long axes, the probable submicroscopic organization of the lipid granules may be determined: the molecules of the lipid constituents determining the optical character of the granules must be radially oriented in the same way as the lipids in the myelin sheath or in myelin figures (SCHMIDT, 1937, SCHMITT, 1939). The experiments on the lipids extracted from the granules seem to lend support to this assumption. The formation of spherites at rubbing in water can hardly be explained except on the basis of the presence of lipid molecules with polar groups (cf. BEAR, PALMER and SCHMITT, 1941, PALMER and SCHMITT, 1941, SCHMITT, BEAR and PALMER, 1941). Such molecules have a strong tendency to form bimolecular layers which in spherites must be concentrically wrapped. Such an organization in the spherites from the extracted lipid granules offers a direct explanation of their negative birefringence when at first built up from a chloroform solution, and of the reversal to positive character at further evaporation of the solvent. An incorporation of chloroform between bimolecular layers of lipid molecules would cause a negative lamellar birefringence in the spherites (cf. NAGEOTTE, 1931) and after the evaporation of the chloroform this would be replaced by a positive intrinsic double refraction, just as observed. (An incorporation of benzene in Na-oleate micelles has been demonstrated by X-ray diffraction measurements by KIESSIG and PHILIPPOFF, 1939.)

The value of the double refraction obtained for the lipid

granules, 0.04, is remarkably high. This shows that the lipid molecules have a high degree of orientation. The myelin sheath, for instance, which by X-ray diffraction studies has been shown to have the lipid molecules well oriented in the radial direction, has a value of the birefringence approximately 0.011 (SCHMITT and BEAR, 1937). As a matter of fact the found value (0.04) for the granules approaches the values of birefringence for fatty acids and cholesterol in the crystalline state (cf. WINCHELL, 1943); BLODGETT and LANGMUIR (1937) found a value of 0.06 for monolayers of barium stearate.

The investigation has thus shown that the lipid granules have a molecular organization of high structural regularity comparable with the crystalline state. As can be computed from Table I (section III) the lipid granules contain approx. 74 per cent esterified cholesterol, 23 per cent residual fatty acids, 2 per cent phospholipids and 1 per cent non-esterified cholesterol. In consideration of the amounts of fatty acids which can be bound to the esterified cholesterol, the cholesterol esters will amount to more than 80 per cent of the total lipids in the granules. The conclusion must be drawn that the double refraction of the lipid granules is mainly due to these cholesterol esters. WHITE (1907—08), CRAVEN-MOORE (1907—08), and DERVICHIAN and MAGNANT (1946) have demonstrated that, together with fatty acids, the cholesterol esters are able to form myelin figures. The low content of phospholipids seems to explain the fact that the lipid granules neither swell nor disintegrate in water, which, on the other hand, is the case concerning the myelin sheath and in this structure is due to a high content of phospholipids, especially cephalin (PALMER and SCHMITT, 1941, SPIEGEL-ADOLF and HENNY, 1947).

III. *The distribution of lipids in the cytoplasm of the interstitial gland cell.*

In each of the experiments I—VI, Tables I and II, 8 pseudopregnant rabbits were used, in experiments I—III unstimulated animals and in experiments IV—VI animals stimulated with 450 I. U. PMSG for 12 hours.

The ovaries were homogenized in 0.3 M sucrose, and the homogenates fractionated by differential centrifugation. Four fractions were examined with regard to their lipid distribution in the cytoplasm:

1. Whole cytoplasmic fraction. This fraction contains all cytoplasmic components apart from the cell nuclei. — The subsequent fractions are subfractions of this "whole cytoplasmic fraction", and at calculation of the lipid distribution the lipid content of each subfraction is expressed in per cent of the content in the whole cytoplasmic fraction.

2. Lipid granules.

3. Large granules. This fraction contains the particles sedimenting at the final centrifugation. Practically all the particles visible in light field microscope are recovered in this sediment. In all probability, however, this fraction is contaminated with microsomes adhering to the large granules. — On account of the separation technique this fraction of large granules may be expected to contain the mitochondria. Smears of this fraction were fixed with potassium bichromate-formalin according to Regaud and stained with aniline acid fuchsin according to Altmann. The large granules were found to be fuchsinophilic, as are mitochondria in tissue sections.

4. Remaining cytoplasmic fraction. This fraction contains the ground cytoplasm, the microsomes and an insignificant contamination with particles, visible in the light field microscope. A small flushy layer on the top of the large granule sediment was withdrawn together with the supernatant and was included in the remaining cytoplasmic fraction.

Unstimulated ovaries. As is evident from Table I, practically the whole quantity of esterified cholesterol (95 per cent) was found in the lipid granules. On the whole this was the case also concerning residual fatty acids (91 per cent). The non-esterified cholesterol, on the other hand, was mainly found outside the lipid granules (26 per cent in the lipid granules). The phospholipids were the only lipid constituent practically exclusively localized to the large granules and the remaining cytoplasm (36 and 56 per cent respectively).

TABLE I

Experiment No.	Weight of Ovaries Mg.	Isolated Fractions (10.0 ml. homogenate)	Total Cholesterol		Esterified Cholesterol		Free Cholesterol		Phospholipids		Total Fatty Acids		Residual Fatty Acids	
			Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent
I	3191	Whole cytoplasmic fraction	78.2		74.2		4.0		40.5		107.7		31.1	
		Lipid granules	72.0	92.1	70.7	95.3	1.3	32.5	4.5	11.1	73.7	68.4	23.6	75.9
		Large granules	1.2	1.5	0.20	0.3	0.92	23.0	13.3	32.8	11.0	10.2	2.0	6.4
		Remaining cytoplasm	2.1	2.7	0.92	1.2	1.2	30.0	21.3	52.6	20.7	19.2	5.9	19.0
II	4204	Whole cytoplasmic fraction	118.4		111.8		6.6		61.7		151.0		35.4	
		Lipid granules	108.1	91.3	106.2	95.0	1.9	28.8	3.6	5.8	109 3	72.4	36.2	102.3
		Large granules	2.4	2.0	0.51	0.5	1.9	28.8	24.9	40.4	18.9	12.5	1.9	5.4
		Remaining cytoplasm	5.6	4.7	3.3	3.0	2.4	36.4	34.0	55.1	25.1	16.6	0.22	0.6
III	4235	Whole cytoplasmic fraction	130.8		124.2		6.5		62.8		156.1		31.5	
		Lipid granules	119.0	91.0	117.6	95.0	1.1	16.9	1.2	1.9	108.3	69.4	29.6	94.0
		Large granules	2.6	2.0	1.0	0.8	1.6	24.6	21.0	33.4	16.0	10.2	1.3	4.1
		Remaining cytoplasm	5.4	4.1	2.3	1.9	3.1	47.7	38.7	61.6	30.2	19.3	2.8	8.9
Lipid distribution Per cent of the whole cytoplasmic		Lipid granules	M. =	91.5		95.1		26.1		6.3		70.1		90.7
		Large granules	M. =	1.8		0.5		25.5		35.5		11.0		5.3
		Remaining cytoplasm	M. =											

| Experiment | Weight of | Isolated Fractions | Total Cholesterol | | Esterified Cholesterol | | Free Cholesterol | | Phospholipids | | Total Fatty Acids | | Residual Fatty Acids | |

Experiment No.	Weight of Ovaries Mg.	Isolated Fractions (10.0 ml. homogenate)	Total Cholesterol		Esterified Cholesterol		Free Cholesterol		Phospholipids		Total Fatty Acids		Residual Fatty Acids	
			Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent	Mg.	Per cent
IV	6526	Whole cytoplasmic fraction	13.4		10.8		2.7		47.9		56.7		17.6	
		Lipid granules	8.2	61.2	7.4	68.5	0.78	28.9	3.3	6.9	23.7	41.8	16.4	93.2
		Large granules	1.0	7.5	0.56	5.2	0.44	16.3	10.6	22.1	7.3	12.9	0.0	0.0
		Remaining cytoplasm	3.7	27.6	2.6	24.1	1.1	40.7	36.4	76.0	27.3	48.1	1.3	7.4
V	4971	Whole cytoplasmic fraction	16.8		13.8		3.1		61.7		78.6		28.3	
		Lipid granules	12.0	71.4	11.7	84.8	0.36	11.6	0.1	0.2	29.4	37.4	21.6	76.3
		Large granules	1.1	6.5	0.42	3.0	0.69	22.3	16.7	27.1	13.5	17.2	2.0	7.1
		Remaining cytoplasm	3.4	20.2	1.6	11.6	1.7	54.8	43.0	69.7	33.3	42.4	3.5	12.4
VI	6106	Whole cytoplasmic fraction	14.1		10.8		3.3		91.7		98.8		30.7	
		Lipid granules	10.2	72.3	9.8	90.7	0.37	11.2	1.7	1.9	38.8	39.3	31.7	103.3
		Large granules	0.94	6.7	0.19	1.8	0.76	23.0	24.3	26.5	20.1	20.3	3.8	12.4
		Remaining cytoplasm	3.1	22.0	1.6	14.8	1.5	45.5	64.7	70.6	44.4	44.9	0.22	0.7
Lipid distribution Per cent of the whole cytoplasmic fraction		Lipid granules	M.=	68.3		81.3		17.2		3.0		39.5		90.9
		Large granules	M.=	6.9		3.3		20.5		25.2		16.8		6.5
		Remaining cytoplasm	M.=	23.3		16.8		47.0		72.1		45.1		6.8

The distribution of the lipids *within* the lipid granules can be computed from Table I:

Esterified Cholesterol	Free Cholesterol	Phospholipids	Residual Fatty Acids
74 %	1 %	2 %	23 %

If the fatty acids bound to the esterified cholesterol are included at the calculation of the intragranular lipids, the result will be approx. 83 per cent cholesterol esters.

It is to be noted that non-esterified cholesterol and phospholipids are present in very small amounts in the lipid granules.

The most outstanding fact about the lipid distribution within the cytoplasm of the interstitial gland cells is that no less than approx. 67 per cent of the total lipids (computed from Table I) are localized in specific structures, the lipid granules, and that approx. 83 per cent of their lipid content consists of cholesterol esters.

Stimulated ovaries. There is no possibility to directly compare the absolute amounts of the different lipids (mg./10.0 ml. homogenate), found in Table II, with corresponding values in Table I. The difficulty lies in the fact that the percentage of cell breakage at homogenization of the ovaries is probably not constant and cannot be calculated from the present material.

As is evident from a previous work (CLAESSON, HILLARP and HÖGBERG, 1953) the gonadotrophic stimulation used in the present experiments gives a pronounced reduction in esterified cholesterol (about 80 per cent). The reduction in cholesterol esters must be referred to a disappearance of this cholesterol from the lipid granules. Only very small amounts can be taken from the large granules and the remaining cytoplasm. In spite of the fact that very considerable amounts of esterified cholesterol must have disappeared from the lipid granules, approx. 81 per cent of the total cytoplasmic choesterol esters are still found in the granules after stimulation. The distribution of the different lipids within the lipid granules is a follows:

Esterified Cholesterol	Free Cholesterol	Phospholipids	Residual Fatty Acids
27 %	1 %	5 %	66 %

Approx. 33 per cent of the total cytoplasmic lipids are localized in the lipid granules after the stimulation.

It has not been possible to interpret the increase in phospholipids previosly observed at gonadotrophic stimulation (CLAESSON, DICZFALUSY, HILLARP and HÖGBERG, 1948, CLAESSON, HILLARP and HÖGBERG, 1953, CLAESSON, 1954 b and c). The present material, however, gives a possibility to elucidate this problem, as the intracellular distribution of the phospholipids before and after gonadotrophic stimulation can be judged. As is evident from Tables I and II, the part of the total cytoplasmic phospholipids which belongs to the fraction "remaining cytoplasm" has increased from 56.4 to 72.1 per cent. Student's test has been used to compare the difference between the means of the two series. This calculation gave a statistically significant difference, $0.001 < P < 0.01$.[1]

There is no reason to doubt that in the present material there has occurred an increase in the absolute amounts of phospholipids after the gonadotrophic stimulation (450 I. U. PMSG, 12 hours, 24 animals in all). If there is an increase in phospholipids in the individual interstitial gland cell this increase must have taken place in the fraction "remaining cytoplasm", probably exclusively.

This assumption is in good agreement with the observation that the supernatant, obtained after the final centrifugation of homogenates from the stimulated ovaries, shows a pronounced change. Before the stimulation the supernatant contains only a few particles, visible in the light-field microscope and of very small size. When examined by dark-field illumination these particles are distinct bodies, showing an intense light scattering

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[1] $$t = \frac{m_1 - m_2}{s} \sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}}$$

where

$$s = \sqrt{\frac{\Sigma(x - m_1)^2 + \Sigma(y - m_2)^2}{n_1 + n_2 - 2}}$$

Symbols:

x, y = the value of the individual observation.
n_1, n_2 = the number of observations in the two series.
m_1, m_2 = the arithmetic mean.

contrary to the numerous non-distinct particles with a weak light scattering (probably microsomes). After the stimulation there is an enormous increase in the number of particles with distinct outlines and with intense light scattering power; they are of so small a size that they are hardly visible in the light-field microscope. Of course it is impossible to determine the nature of these particles on the basis of the microscopical examination. But it is most likely that they are new-formed mitochondria, as after gonadotrophic stimulation of the ovaries there is a pronounced increase in the activity of succinic dehydrogenase in the supernatant (HILLARP, 1954), indicating a presence of numerous mitochondria in this fraction — in contrast to the state before stimulation.

The increase in phospholipids at gonadotrophic stimulation, established in previous works, and the increased part of the total cytoplasmic phospholipids in the fraction "remaining cytoplasm", observed in this paper, seem to obtain their explanation on the basis of an increase in cytoplasmic particles, probably mitochondria. A new formation of mitochondria (and microsomes?) ought to cause an increase in the cell phospholipids, as these cellular components contain high concentrations of phospholipids (SWANSON and ARTOM, 1950, KRETCHMER and BARNUM, 1951). The existence of an increase in the number of mitochondria seems to be supported by observations on the effect of ACTH on the adrenal cortex. MILLER (1952) found that the total number of mitochondria, demonstrated in histological preparations, were markedly increased at stimulation with ACTH; PERRY and CUMMING (1952) reported that ACTH caused an increased activity of succinic dehydrogenase.

Thus apparently gonadotrophic hormone, directly or indirectly, acts as a generator for a formation of mitochondria. This is reasonable as mitochondria are the main site for the energy-generating processes in the cell. Furthermore it seems to be evident from the investigations on the steroid synthesis in adrenal cortex that mitochondria are indispensable structures for certain stages of this synthesis (DORFMAN et al., 1953).

General Discussion

It has been shown in tracer experiments that cholesterol can be a precursor of steroid hormones formed in adrenal cortex, placenta and testis (cf. BLOCH, 1950, HECHTER et al., 1951, HECHTER, 1953, GALLAGER et al., 1954, HEARD et al., 1954). But no direct evidence for the biogenesis of oestrogens from cholesterol has been presented. Since BLOCH (1945) established that deuterio-cholesterol could be transformed to pregnandiol, only one experiment on the direct biosynthesis of oestrogens from labeled cholesterol has been published. This experiment (HEARD and O'DONNELL, 1954) did not give any evidence for such a transformation in a pregnant mare. From this experiment, however, it is not possible to conclude that the oestrogens produced in the ovary might not arise from cholesterol. From a preparative and chemical point of view this experiment may be adequate, but other experimental conditions may have been inadequate.

On the basis of the results obtained in investigations on the ovary (CLAESSON, HILLARP et al., 1946—1949, 1953, CLAESSON, 1954) the assumption was made that esterified cholesterol in the ovarian interstitial gland cells is a precursor of oestrogenic hormones. The evidence for the conception of the esterified cholesterol being a precursor in this gland is summarized as follows:

1. The interstitial gland cells of rabbit, rat and guinea-pig ovaries store large amounts of esterified cholesterol at certain functional stages. If calculated as cholesterol esters the storage can amount to as much as 6—7 per cent of the wet weight. No cells but the adrenal cortical cells show this high capacity of storage. — In the rabbit ovary the stored sterol has been isolated and found identical with cholesterol.

2. The esterified cholesterol in the interstitial gland cell undergoes pronounced quantitative changes, ranging from high storage to depletion, which are regulated by pituitary trophic hormones like other steriod hormone producing cell systems, as shown for adrenal cortex (cf. LONG, 1946) and corpus luteum (EVERETT, 1947). — In this connection it is of interest that the

transition from a non-oestrogen producing stage to an oestrogen producing stage in the interstitial gland of the infantile rat ovary coincides with a commencing storage of cholesterol (FALCK, 1953).

3. A gonadotrophic stimulation (450 I. U. PMSG) of the pseudopregnant rabbit ovary produces a rapid and pronounced reduction of the stored esterified cholesterol. Thus also on this point there is a complete agreement with the adrenal cortex. The time course of the cholesterol reduction in the ovary is almost identical with the correspondent reduction in the adrenal cortex at stimulation with ACTH (cf. LONG, 1946). It may be pointed out that the mobilization of cholesterol esters in the ovary runs to completion at a bottom level representing only about 10 per cent of the initial content.

4. There exists a quantitative relationship between the degree of reduction in the stored esterified cholesterol and the dose of injected PMSG. What is more, practically the whole decrease takes place within a narrow range of doses (5—40 I. U.), exactly as ought to be expected from a stored precursor. The esterified cholesterol is also found to react at a lower dose level than do other ovarian lipids, just as it is found to be the earliest responding fraction.

Thus, the most outstanding feature in the ovarian interstitial gland cell is its capacity of storing esterified cholesterol and the pronounced changes in this cholesterol *at the specific stimulation* with gonadotrophic hormone.

In the present work it was found that about 95 per cent of the total cytoplasmic cholesterol esters are localized to lipid granules in the living interstitial gland cell. These granules must be considered to be specific structures for the storage of esterified cholesterol. The molecular arrangement of the lipid within the granules shows a high degree of organization which obviously provides the lipid complex with a structural stability but which also allows a rapid mobilization of the cholesterol. The effect at gonadotrophic stimulation on the lipid granules is first of all such a mobilization, characterized by a rapid reduction in size and birefringence of the granules.

The lipid granules do not exist only in the ovarian interstitial

gland cell but also in other cells, producing steroid hormones (adrenal cortex and corpus luteum, HILLARP, 1954). Hence it may be concluded that the interstitial gland cell has the same specific properties as have the cells of adrenal cortex and corpus luteum, as well from a physiological point of view, regarding the trophic control of the changes in esterified cholesterol, as from a structural point of view, regarding the intracellular localization and organization of the esterified cholesterol in the living cell. These facts present strong evidence for the conception of cholesterol being a stored precursor of the steroid hormone produced in the ovarian interstitial gland at gonadotrophic stimulation.

Summary

The intracellular localization of the lipids in the living interstitial gland cell of the pseudopregnant rabbit ovary has been investigated at two different functional stages, stage of cholesterol storage and stage of cholesterol mobilization at gonadotrophic stimulation with pregnant mare serum gonadotrophin.

The methods for demonstration of lipids in cells after fixation have been shown to be inadequate at studying the intracellular distribution of lipids in the ovary.

The living interstitial cell is heavily loaded with specific lipid granules, which are relatively uniform in size (about 1 μ) and very likely of one type only. These granules show a pronounced stability and hence may be isolated as a pure fraction through differential centrifugation of ovarian homogenates.

The lipid granules have a spherite texture, the character of the double refraction being positive with reference to the radially oriented optical axis. The physical properties of the granules indicate that the lipid molecules are oriented in a radial direction and organized in bimolecular layers concentrically wrapped in the spherites. The value of birefringence is very high, approx. 0.01, thus approaching the values for lipids in the crystalline state. This shows that the lipid molecules have a high degree of orientation in the granules.

At gonadotrophic stimulation the lipid granules show pronounced changes, characterized by a rapid reduction in size and birefringence. These changes are primarily an expression of a mobilisation of esterified cholesterol from the granules.

The lipid distribution within the cytoplasm of the interstitial cell was examined by separation of the different cell components. In unstimulated ovaries approx. 67 per cent of the total cytoplasmic lipids, approx. 95 per cent of the esterified cholesterol and approx. 91 per cent of the residual fatty acids are localized in the lipid granules. The distribution of the lipids within the granules is: 74 per cent esterified cholesterol (83 per cent if expressed as cholesterol esters), 1 per cent non-esterified cholesterol, 2 per cent phospholipids and 23 per cent residual fatty acids. The corresponding values after gonadotrophic stimulation (450 I. U. PMSG, 12 hours) are: 27, 1, 5, and 66 per cent respectively. Approx. 33 per cent of the total cytoplasmic lipids are still localized in the lipid granules after stimulation.

Evidence is presented for an increase in cytoplasmic phospholipids at gonadotrophic stimulation and for the assumption that this is an expression of a new formation of cytoplasmic particles, probably mitochondria.

The evidence for the conception of esterified cholesterol being a precursor of the steroid hormone produced in the ovarian interstitial cell is summarized. The conception that the lipid granules are specific structures for the storage and mobilization of this cholesterol is discussed.

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The structure of the adrenaline and noradrenaline containing granules in the adrenal medullary cells with reference to the storage and release of the sympathomimetic amines[1]

By

NILS-ÅKE HILLARP AND BODIL NILSON

In previous works (HILLARP and NILSON, 1953, 1954, HILLARP, LAGERSTEDT and NILSON, 1953, HILLARP, HÖKFELT and NILSON, 1954), it has been shown that adrenaline and noradrenaline in the adrenal medullary cell are stored in special granules which can be isolated as a practically pure fraction, and the generale chemical composition of these granules has been examined.

A further investigation about the structure of the medullary granules seemed, however, necessary. Such an investigation is likely to give data that ought to be of value to the solution of two important problems concerning the medullary cell: how the large quantities of catechols[2] are stored and how these are released at stimulation.

At the isolation experiments the medullary granules showed to be relatively stable structures. A rapid liberation of catechols was, however, observed at treatment of the granules with physical and chemical agents of quite different nature. It seemed probable that a further examination of this phenomenon might give valuable information on the structure of the granules.

[1] Aided by a grant from Statens Medicinska Forskningsråd.

[2] For simplicity's sake the term 'catechols' is used for adrenaline + noradrenaline.

Material and Methods

The specific medullary granules from homogenized cow adrenal medulla were isolated by differential centrifugation in 0.25—0.9 M sucrose solution with a technique described in previous papers. At every separate experiment granules from 4—10 adrenals were used. The granules isolated from different samples of adrenals contained 77—82 per cent of the total catechol amounts in the different homogenates.

The medullary granules were isolated from the various homogenates by centrifugation in a Wifug R angle head centrifuge at 6800 × g during 30 minutes. This gives a complete sedimentation of all the granules containing catechols and therefore it is easy to withdraw the supernatant by a capillary pipette. Where it is not otherwise mentioned these unwashed granules were used directly in the various experiments. — The centrifugations were made at +4 to +8° C. or at room temperature.

At suspension of granules sedimented by centrifugation a capillary pipette with an inner diameter of about 0.5 mm. was used. This suspension technique gives a catechol release that is only 6—9 per cent of the total amount of catechols in the sediment. Hence, in the various experiments, the release observed at treatment with different agents has been corrected on the basis of a suspension release of 9 per cent in order to obtain the real release of catechols caused by the treatment.

The amounts of released catechols were calculated by determination of the catechol content in the supernatant after the granules had been sedimented by centrifugation (Wifug R angle head centrifuge; 6800 × g for 20—30 min. according to the density of the suspension medium).

The determination of adrenaline and noradrenaline were performed colorimetrically according to Euler and Hamberg (1949). The protein determinations were made according to the micro-Kjeldahl procedure after trichloracetic acid precipitition and lipid extraction. Further information concerning the performance of these determinations are found in a previous paper (Hillarp and Nilson, 1954).

Determinations of the pH of the various solutions were made electrometrically with a glass electrode.

Experimental results

I. *Physical and chemical agents giving release of catechols from the granules*

1. *The effect of tonicity of the suspension medium*

Granules isolated in 0.25 M sucrose, can be suspended in sucrose or electrolyte solutions (e. g. NaCl, KCl) of the same or higher osmolarity without a release of catechols from granules. If, on the contrary, the tonicity is decreased there occurs a liberation, the magnitude of which is due to the concentration difference. This is shown in Fig. 1, which illustrates the percental release from granules isolated in 0.25 M sucrose and thereafter transferred to 0.20, 0.15, 0.10 or 0.05 M sucrose for 1 hour at $+3^{\circ}$ C. Already at lowering of the molarity to 0.20 more than 50 per cent of the catechols were liberated and the release was practically completed in 0.15 M sucrose.

This release at lowering of the tonicity is rapid. It takes place before the sedimentation of the suspended granules by centrifugation (20 min.) is accomplished, and therefore it is not possible to follow the time course by the technique applied. It appeared, however, that the release was as high after 30—60 min. as after 24 hours. Thus there occurs a rapid initial release up to a certain value where a plateau is reached and no further liberation takes place. Hence this corresponds to the haemolysis of the red blood corpuscles under the same conditions.

However, a release of catechols does not only occur at the transfer of granules from approx. isotonic to hypotonic solutions. On the contrary, it appeared that a lowering of the solute concentration of the suspension medium gives a release whether this lowering makes the medium hypotonic or not. This is evident from Figs. 2 and 3. The experiments in Fig. 2 shows that if granules isolated in a 0.8 M sucrose solution were transferred to 0.5 M sucrose, a release of more than 50 per cent occurred. The liberation of catechols was practically complete at the lowering of the sucrose concentration to isosmolarity. — This phenomenon is independent of the suspension medium being a non-electrolyte as sucrose or an electrolyte, e. g. KCl.

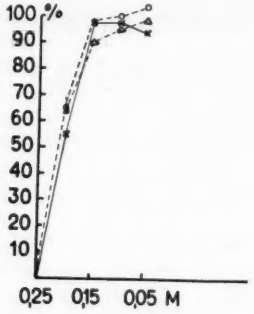

Fig. 1.

Figs. 1—3. Per cent release of catechols from medullary granules at lowering of sucrose concentration in the suspension medium. The granules were isolated in 0.25, 0.80 and 0.40 M sucrose respectively and transferred to sucrose solutions of lower molarity for 1 hour at + 3° C.

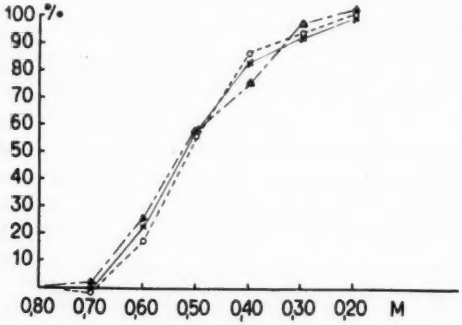

Fig. 2.

The adrenaline and noradrenaline released from granules at lowering of the solute concentration exist in all probability in real solution in the suspension medium. This is shown by experiments with two different methods:

Granules isolated in 0.3 M sucrose were suspended in water after which they were sedimented at 6800 × g for 60 min. (5° C.). A determination of the amount of cathechols in the supernatant showed that this contained the whole original amount of cate-

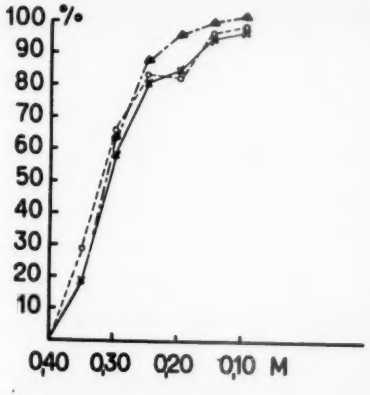

Fig. 3.

chols in the granules. The supernatant was then centrifuged at 148.000 × g for 60 min. (Spinco centrifuge, approx. + 5° C.). A small sediment was formed but the whole amount of catechols was recovered in the supernatant. — This experiment, however, does not quite exclude that the catechols might be bound to non-sedimentable particles of very small dimensions. But strong reasons against such an assumption were obtained at an examination of the supernatant with paper electroforesis (to be published). These experiments showed that adrenaline as well as noradrenaline moved quite independent of the proteins in the supernatant and in the same manner as the pure substances in solution.

2. *The effect of pH of the suspension medium*

Granule suspensions in 0.3—0.8 M sucrose have a pH of 6.4—6.6. Any significant release of catechols does not occur from granules in such suspension for 24—48 hours at + 3° C.

At a pH below 6, however, granules are no longer stable structures. At suspension of granules (isolated in 0.9 M sucrose) in N acetic acid acetate buffer pH 6.0—4.0 for 1 hour at + 3° C. no liberation of catechols occurred at pH 6.0 and only an inconsiderable liberation at pH 5.5 (Fig. 4). At pH 5.0, on the other

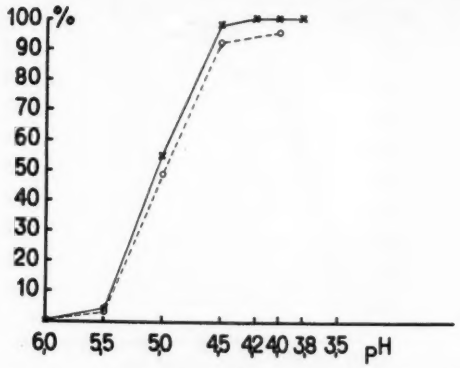

Fig. 4. Per cent release of catechols from medullary granules at lowering of pH of suspension medium. Granules isolated in 0.9 M sucrose were transferred to N acetic acid acetate buffer at + 3° C. for 1 hour.

hand, about 50 per cent of the catechols were released and the release was practically complete at pH 4.5.

Thus a rapid release of catechols occurs at or below pH 5, but this does not have the same time course as at lowering of the tonicity of the suspension medium. Here occurs, on the contrary, a release proceeding in time. This is evident from Fig. 5, which shows that granules, suspended in acetate buffer pH 5.0 completely liberate their catechols within 4 hours. Fig. 6 shows that a marked release occurs at pH 5.5, too, if the treatment is extended to 24 hours.

Control experiments with granule suspensions in sucrose solution, adjusted to different pH by acetic acid, gave the same result as the experiments with acetic acid acetate buffer. This shows that the liberation in this buffer is a function of pH and not quite simply dependant on a permeability to anions or to free acid of a granule membrane. That the catechol release is no specific effect of acetic acid is evident from the fact that for instance citric acid in sucrose solution, has the same effect.

No release of catechols was demonstrable from granules (isolated in 0.3 M sucrose), which were suspended for 1 hour in $^1/_3$ M KH_2PO_4-Na_2HPO_4-buffer of 5.4—8.0 or for 24 hours in borate

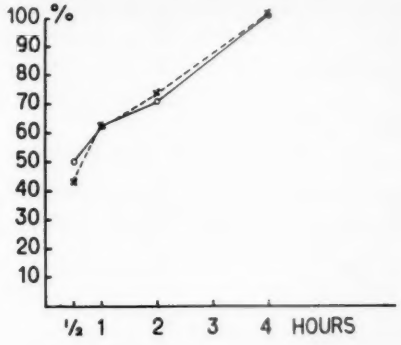

Fig. 5. Per cent release of catechols from medullary granules suspended in N acetic acid acetate buffer pH 5.0.

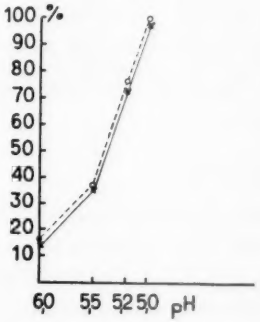

Fig. 6. Per cent release of catechols from medullary granules treated as in Fig. 4. Time: 24 hours.

buffer pH 7.0—7.5 (0.1 M boric in 0.3 M sucrose, adjusted by 0.05 M Na-borate). The effect of higher pH values was not investigated owing to the rapid oxidation of adrenaline and noradrenaline at alkaline reaction.

The granules showed a marked tendency to agglutinate at a pH approaching 5. In acetic acid acetate buffer the agglutination was pronounced at pH 5.2—3.8.

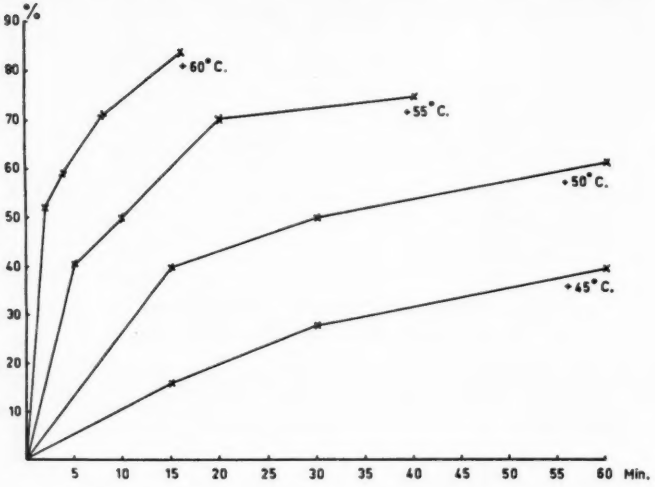

Fig. 7. Per cent release of catechols from medullary granules in 0.3 M sucrose at different temperatures. Average values from three experiments with granules isolated from three different homogenates.

3. *The effect of temperature*

It is possible to keep medullary granules in 0.25—0.9 M sucrose at +3° C. for several days without having any liberation of catechols. At +20° to +30° C. they are stable for several hours.

At temperatures above +45° C. the catechols are released at a high rate which is shown in Fig. 7. From three different granule suspensions in 0.3 M sucrose 50 per cent were released within 2, 10 and 30 min. at +60°, +55° and +50° C. respectively. The release was complete after some minutes at +70° C., on account of which the time course could not be followed.

The technique applied (heating in a vessel with thin walls on water-bath of constant temp. and thereafter rapid cooling by ice-water) allows no closer analysis of the liberation course, but it is evident from Fig. 7, that the increase in the reaction velocity was not obviously greater from +50° to +60° C. than from +45° to +55° C.

Freezing, too, gives a release of catechols. At freezing of granules, suspended in 0.3 M sucrose, with CO_2-snow for 5 min. 69—75 per cent of the catechols were found in the suspension medium after thawing (5-experiments).

4. *The effect of detergents and digitonin*

Anionic as well as cationic detergents give a complete liberation of catechols from the granules in low concentrations (Table I and Fig. 8.)

TABLE I.

Release of catechols from medullary granules at different concentrations of cetyl pyridinium bromide and of sodium lauryl sulfonate. Granules isolated in 0.3 M sucrose were suspended in 2.0 ml. of detergent solution in 0.3 M sucrose for 1 hour.

	Amounts of granules Mg. wet weight	Release of catechols at different concentrations of the detergent		
		10^{-2} M	10^{-3} M	10^{-4} M
I. Cetylpyridinium bromide	41 mg.	100%	92%	7%
	40	102	91	4
	33	103	95	9
	33	100	93	5
	32	100	90	3
II. Na-lauryl sulfonate	47	82	4	2
	35	91	5	4
	38	95	5	3
	36	95	3	1

In these experiments granules (30—50 mg wet weight, isolated in 0.3 M sucrose) were suspended in 2.0 ml 10^{-2} to 10^{-4} M cetylpyridinium bromide, sodium lauryl sulfonate or sodium dodecyl sulfate in 0.3 M sucrose. After 1 hour at $+3°$ C. the catechol content in the suspension medium was determined.

SDS and CPB produced a 90—95 per cent release in 1.6×10^{-3} M and 10^{-3} M solutions respectively, while sodium lauryl sulfonate

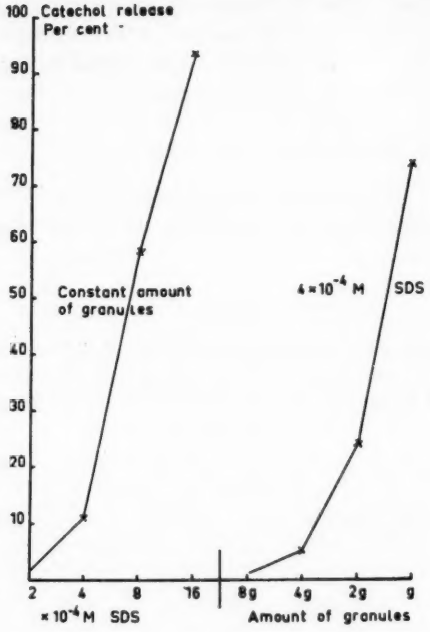

Fig. 8. Per cent release of catechols from medullary granules at different concentrations of sodium dodecyl sulfate in 2.0 ml. 0.3 M sucrose (left curve; amount of granules approx. 35 mg. wet weight) or at different amounts of granules in 4×10^{-4} M SDS (right curve; 4G approx. 35 mg. wet weight). 1 hour at + 3° C.

had this effect only in 10^{-2} M solution. As at protein denaturation by detergents, however, the effect on granules was not directly dependent on the actual detergent concentration but instead on the relation between the amounts of detergent and of granules respectively. This is evident from Fig. 8, which shows that the release from a constant amount of granules became greater at an increased SDS-concentration but also that the same effect was attained by decreasing the amount of granules in a constant SDS-concentration.

A similar experiment with digitonin gave the same result as

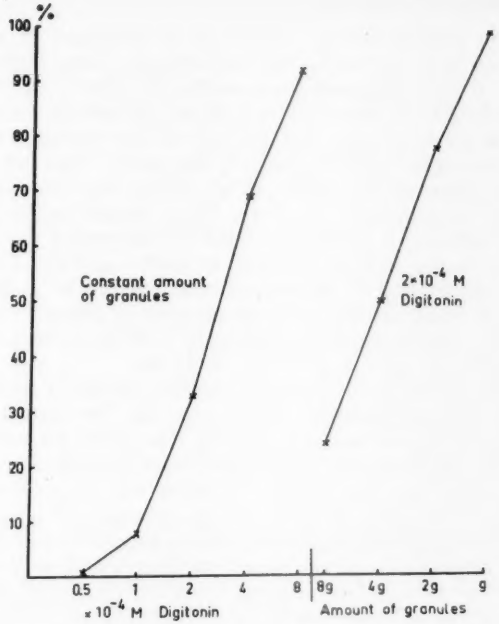

Fig. 9. Per cent release of catechols from medullary granules treated with digitonin in the same way as with SDS in Fig. 8.

with detergents (Fig. 9). Granules (33—50 mg wet weight) from three different homogenates were suspended in 2.0 ml. 0.5—8 × 10^{-4} M digitonin in 0.3 M sucrose. As at using detergents the release of catechols was dependent on the amount of granules in relation to the amount of digitonin and not on the actual digitonin concentration.

The release from granules was as great after one hour as after 12 hours in digitonin. Thus, as at a lowering of the tonicity of suspension medium, digitonin gives a release that rapidly reaches a certain value but does not show a further progress after that.

5. *The effect of lecithinase A*

The used preparation of lecithinase A consisted of a highly active, dried protein fraction from an extract of honey bee venom.[1] A sample of this preparation was extracted with $^1/_5$ M boric acid borate buffer pH 7.5 according to Palitsch, containing 0.01 M $CaCl_2$, and was kept at $+3°$ C. The activity of the extract was tested through determination of its haemolyzing power. Varying amounts of the lecithinase extract were put to a 2 per cent blood corpuscle suspension (human red cells, three times washed in 0.85 per cent NaCl) in buffered 0.85 per cent NaCl (4 volumes of 0.85 per cent NaCl + 1 volume of borate buffer as above) and the haemolysis was photoelectrically determined after incubation at $+30°$ C. for 30 min. In these experiments, an amount of extract containing 4μg. of the dry protein fraction, gave a 50 per cent haemolysis of 1.01×10^9 red cells in a volume of 5.0 ml.

Lysolecithin was made from egg lecithin prepared according to MacFarlane and Knight (1941). One ml. lecithinase extract, containing 10 mg. of the dry protein, was put to an emulsion of 15 mg. lecithin in 1 ml. water. Incubation was made during 24 hours at $+38°$ C. The lecithin emulsion was then extracted by ether which was discarded and 10 ml. ethanol was added to the water solution. The precipitated proteins were removed by centrifugation after which the solution was evaporated *in vacuo* (N_2) at $+40°$ to $+45°$ C. to approx. 2 ml. 50 ml. ethyl. ether was added and the solution was kept at $+3°$ C. for 24 hours. The ether was discarded and the ether-insoluble residue was — after drying — dissolved in 10 ml. 0.3 M sucrose. This extract contained, in all probability, also some small amounts of lysocephalin (cf. Levene, Rolf and Simms, 1924).

The lecithinase effect on the medullary granules was examined in the following manner. Granules, isolated in 0.3 M sucrose from four different homogenates, were washed three times in 0.3 M sucrose in order to remove all remaining cytoplasm. Equal amounts of these granules (approx. 40 mg. wet weight) were suspended in 2.0 ml. 0.3 M sucrose, containing varying amounts of lecithinase A extract, and were incubated together with controls in borate-buffered sucrose at $+38°$ C. for 30 min. The granules were then sedimented by centrifugation in the cold and the liberation of catechols was determined. — The effect of lysolecithin was

[1] The preparation of lecithinase was produced and kindly put at our disposal by A-B Leo, Helsingborg.

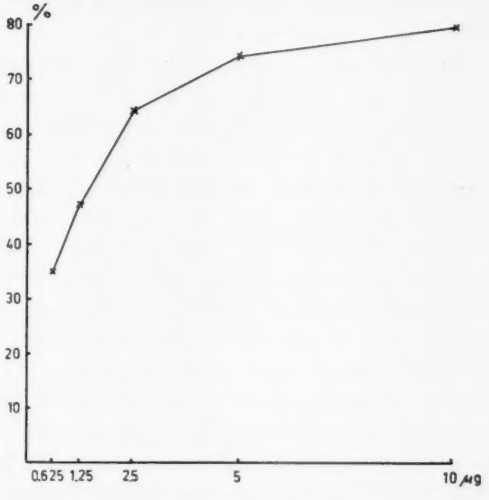

Fig. 10. Per cent release of catechols from medullary granules at different concentrations of lecithinase A. Washed granules (approx. 40 mg. wet weight) were suspended in 2.0 ml. 0.3 M sucrose containing the enzyme and incubated for 30 min. at + 30° C. Average values from two experiments.

examined by suspending granules in 2.0 ml. 0.3 M sucrose, containing various concentrations of the lysolecithin extract. The catechol release was determined after incubation during 30 min. at + 4° C.

As is shown by Fig. 10, already 2 µg. of the lecithinase preparation give a more than 50 per cent release of catechols from approx. 40 mg. granules. There was no release in the controls without lecithinase.

The lysolecithin extract had the same effect as lecithinase A and gave a marked release in dilutions down to 1 : 100. In control experiments with lecithin only and with lecithin, incubated and extracted in the same way as at the preparation of lysolecithin, no release was observed.

II. *Catechol release and the distribution of adrenaline and noradrenaline*

The medullary granules isolated from cow adrenals contain beside adrenaline also noradrenaline which amounts to 20—30 per cent of the total catechols (HILLARP and NILSON, 1954). These two substances must be stored in such a way that certain granules contain only (or mainly) noradrenaline and the other ones adrenaline. This is evident from the fact that the medullary granules are derived from two different types of cells, which store adrenaline and noradrenaline respectively (HILLARP and HÖKFELT, 1953, 1954). If the granules are not only structures for storage but also the site of the synthesis of the catechols, the two types of granules must differ concerning the synthesis system. Hence an investigation on the release of catechols at various treatments of the granules must pay regard to the possibility that the two types do not react in the same manner on account of differences in structure. But it does not seem probable that any essential differences in structure should exist in two formations, so closely related from a functional point of view.

The release of adrenaline as well as noradrenaline from the granules has been examined at three different treatments by determining the distribution of these two substances in the total fraction of liberated catechols. Granules, isolated from seven different homogenates in 0.8 M sucrose, were used in the three experiments.

The following experimental conditions for the catechol release were used:

A. Lowering of the tonicity of the suspension medium, 0.8→0.5 M sucrose.
B. Lowering of pH of the suspension medium. The granules were transferred from 0.8 M sucrose to N acetic acid acetate buffer pH 5.0 and were kept at +3° C. for one hour.
C. Increased temperature. The granules were kept at +37° C. during 2 hours in 0.8 M sucrose.

The results are put together in Table II. In all the cases the released catechol fraction had — within experimental errors —

TABLE II.

The distribution of adrenaline and noradrenaline in the catechol fraction released from medullary granules under different conditions.

Homogenate 0.8 M sucrose	Catechol content of granules isolated from 1.0 ml.	Noradrenaline. Per cent of the catechols	A. Granules transferred to 0.5 M sucrose		B. Granules transferred to buffer solution pH 5.0		C. Granules kept at +37° C. 2 hours	
			Release of catechols	Noradrenaline. Per cent of the released catechols	Release of catechols	Noradrenaline. Per cent of the released catechols	Release of catechols	Noradrenaline. Per cent of the released catechols
I.	977 µg.	22%	59%	27%	69%	19%	31%	23%
II.	1077	26	51	22	67	21	29	25
III.	1436	22	52	23	64	19	31	21
IV.	1872	23	52	29	59	25	28	25
V.	1203	25	49	28	58	25	30	26
VI.	1239	30	49	29	57	24	27	22
VII.	1167	28	49	27	57	23	28	25

the same percental distribution of noradrenaline and adrenaline as the original, total amount of catechols in the granules before the experiments. Thus, the two types of granules reacted in the same way.

As the treatments of the granules were performed with three agents of quite different nature the experiments speak in favour for the assumption that there exist no essential differences in structure between the granules containing noradrenaline and those containing adrenaline, which in fact has been pointed out as probable owing to their functional relations. Nor has it been possible to separate the two types of granules from each other through differential centrifugation (HILLARP and NILSON, 1954).

III. *Changes in optical properties of the granules and liberation of proteins at treatment with agents giving a catechol release*

In order to get further possibilities to interpret the nature of the changes leading to release of catechols at treatment of medullary granules with different agents, experiments were made starting from the hypothesis that the release is a lysis dependent on injuries to a hypothetic granule membrane. — That being the case, the treatment ought to give certain characteristic optical changes in suspended granules and also, in all probability, a release of proteins from the granules.

Medullary granules in suspension show a strong light scattering power, which may be expected to alter at changes in size and structure of the granules. The factors determining the magnitude of light scatter naturally are so complex that there is no possibility to put its absolute values in direct numerical relation to other measurable changes in the granules, e. g. release of catechols. However, optical measurings may give valuable data showing *that* structural changes occur in the granules and showing if these changes are reversible or not and their course in time.

In the experiments performed the light scatter was not directly measured but instead the optical density of the suspensions in 1 cm. cuvettes at 540 mμ with the different media as reference solution. The concentrations of granules were 10—20 mg. (wet

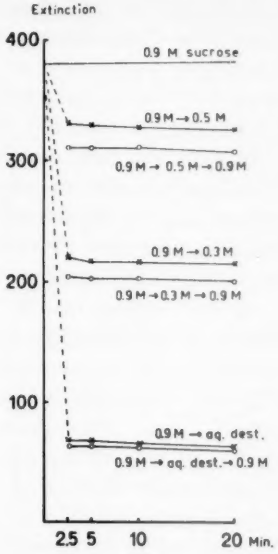

Fig. 11. Changes in optical density of granule suspensions in sucrose solutions at varying of sucrose concentration. The granules were isolated in 0.9 M sucrose. For details, see section III.

weight) in a volume of 5—10 ml. The same granule concentration was always used within each experiment as the optical density of the suspensions do not follow the Beer-Lambert law.

If granules isolated in 0.3—0.9 M sucrose were suspended in water the density decreased to very low values usually to 15—20 per cent of the original density in sucrose (Fig. 11). This decrease had a rapid initial course and was practically completed within 2.5 minutes (Fig. 11). Thereafter no certain changes occurred during 20—40 min.

The changes in granules, which gave a decrease in their light scattering power at lowering of the solute concentration of the medium, proved to be quite irreversible. This is demonstrated in Fig. 11. In this experiment the same amounts of granules (isolated in 0.9 M sucrose) were suspended as well in 10.0 ml.

as in 2.0 ml. 0.9, 0.5, 0.3 M sucrose and water respectively. The density of the suspensions in 10 ml. was measured after 2.5—20 min. 8.0 ml. sucrose solutions were added to the suspensions in 2.0 ml. after 20 min. at +3° C., so that the final concentration was 0.9 M in all the suspensions and the densities were then measured after 2.5—20 min. The light scattering power of the granules, however, did not increase at all when the sucrose concentration was raised back to the original.

The medullary granules undergo irreversible changes also when increasing the sucrose concentration if they have been isolated in approx. isotonic sucrose (0.3 M). This is shown in Fig. 12. In this experiment we used the same method as in previous but granules were isolated in 0.3 M sucros. The effect of 0.9 M sucrose for 2.5 and 10 min. respectively was examined by lowering the concentration to the original and the density was then measured after 2.5—40 min. When raising the concentration from 0.3 to 0.9 M the density showed an increase of approx. 10 per cent which, in all probability, is the summarized effect of several different factors, partly counteracting each other (e. g. decrease in size and dehydration of the granules, increase of the refractive index of the medium). But at the thereafter following lowering of the concentration the extinction values, within 2.5 min., dropped far below the original extinction value for the suspension in 0.3 M solution. A comparison between Figs. 11 and 12 show that, actually, the optical density fell to the same great extent as in the previous experiment with reversed conditions. Fig. 12 shows too that the changes in granules appeared very rapidly (within few minutes) under the influence of 0.9 M sucrose.

This is naturally applicable only to that or those essential changes which lead to the marked decrease in light scattering power at lowering the sucrose concentration.

In order to investigate if possibly the catechol release and the decrease in optical density at lowering of the solute concentration of the suspension medium is correlated in time the following experiment was made. The same amounts of granules, isolated in 0.8 M sucrose, were suspended in 11.0 ml. 0.5 M (suspension A) and 0.3 M (suspension B) sucrose respectively. After

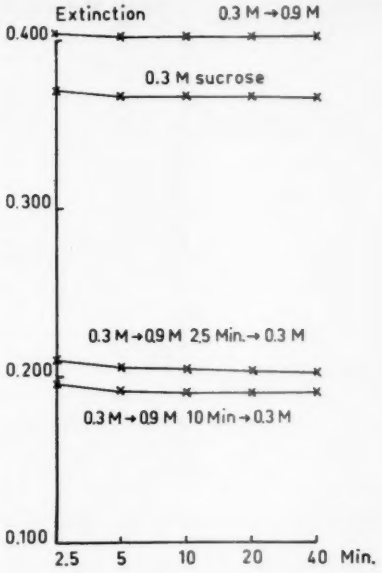

Fig. 12. Changes in optical density of granule suspensions in sucrose at varying of sucrose concentration. The granules were isolated in 0.3 M sucrose. For details, see section III.

2.5, 5, 10, 20 and 40 min. respectively 1.0 ml. was transferred to 9.0 ml. sucrose of the same concentration, and the optical density was measured. At the same time 1.0 ml. of suspension A and B respectively was transferred to centrifuge tubes, containing a quantity of 1.8 M sucrose sufficient to make a final 0.8 M sucrose concentration. The catechol release was determined after centrifugation.

The results are found in Fig. 13. This shows that the changes in the granules giving a decrease in their light scattering power — as was expected — was finished within 2—5 min. and that changes leading to a release of catechols also must have taken place within the very first minutes. These two processes are thus very rapidly accomplished immediately after lowering of the

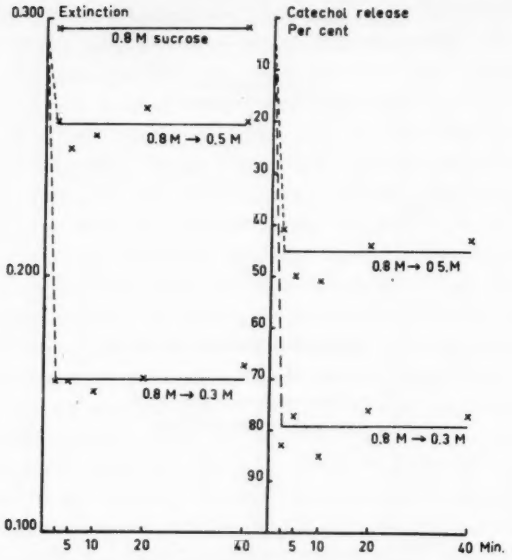

Fig. 13. Changes in optical density and release of catechols at lowering of sucrose concentration. The granules were isolated in 0.8 M sucrose. For details, see section III.

sucrose concentration. But the experiment cannot show if the actual release, too, occurred within this first time lapse.

A lowering of the solute concentration of the suspension medium does not, however, give only the above mentioned changes in the optical properties of granules and a release of catechols but also liberation of great amounts of proteins from the granules.

The release of as well proteins as catechols from the same granules was examined in three different homogenates in 0.8 M sucrose. Granules (washed once in 0.8 M sucrose) were suspended in 0.8, 0.5 and 0.3 M sucrose and water respectively. After 30 min. at $+3^{\circ}$ C. the suspensions were centrifuged in the cold for 60 min. and the contents of proteins and catechols were determined in the supernatant. — This centrifugation is sufficient to sediment all the granules that are microscopically observable (light

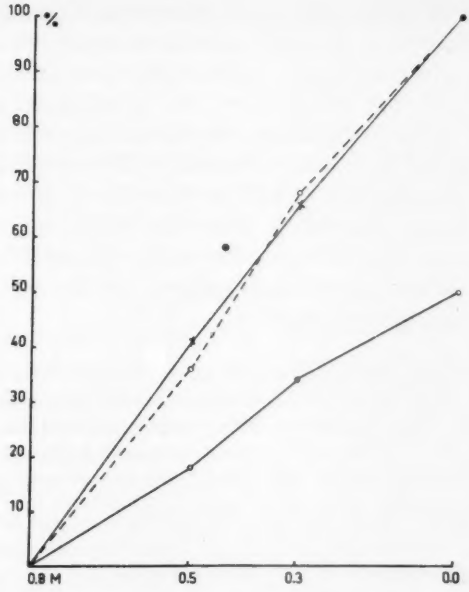

Fig. 14. Per cent release of catechols (x———x) and of proteins from medullary granules at lowering of sucrose concentration. Granules isolated in 0.8 M sucrose were transferred to 0.8, 0.5 and 0.3 M sucrose and water respectively (30 min. at + 3° C.). Average values from three experiments with granules from three different homogenates. o———o protein release in per cent of total proteins in granules. o— — —o protein release in per cent of 'water-soluble' proteins in granules.

and dark field examination). Thus, the proteins found in the supernatant, cannot be present in granules which have not been able to sediment, e. g. on account of swelling.

If the supernatant obtained after centrifugation of granules in a water suspension, is centrifuged at 148.000 × g. for one hour, a small amount of proteins is sedimented. This indicates that a part of the released proteins is particulate. However, it is irrelevant for this investigation if the proteins are liberated in a soluble or a particulate form.

The curve o———o in Fig. 14 represents the release of proteins in per cent of the total proteins. As is evident from this

figure half of the proteins leave the granules in water. If the protein release in 0.5 and 0.3 M sucrose respectively is expressed in per cent of these proteins (representing the maximally releasable proteins), the curve o———o is obtained. This curve shows that there is a good correspondence between the release of catechols and of proteins in 0.5 and 0.3 M sucrose. Apparently the same amounts of catechols and proteins in per cent of the amounts totally releasable in water thus have been liberated at lowering of the sucrose concentration. It is not possible to decide — on the basis of these determinations — if the two curves are corresponding in other respects.[1]

The proteins, leaving granules at lowering of the solute concentration of the suspension medium, naturally give a light scatter. But this is insignificant and barely measurable in the concentrations of granules being used, which appeared from measurings of the optical density after sedimenting the granules by centrifugation. Consequently, this factor cannot complicate the interpretation (see Disc.) of the optical changes. But a factor of greater importance, which might invalidate this interpretation, is the unavoidable changes the refractive index in the experiments mentioned above. These changes must influence the magnitude of the observed values in the optical density, since the light scatter is due to the difference in refractivity between granules and medium. However, Figs. 11 and 12 show that structural changes in the granules play the most important rôle. Otherwise, the suspensions after the procedures $0.9 \rightarrow 0.3$ M and $0.9 \rightarrow 0.3 \rightarrow 0.9$ M (Fig. 11) could not have had the same approx. density. Nor would a suspension after the operation $0.3 \rightarrow 0.9 \rightarrow 0.3$ M show the observed density, considerably lower than the suspensions in 0.3 M sucrose (Fig. 12). Further, it may be pointed out that if the experiments with lowering of the solute concentration of the suspension medium are carried out in the presence of 0.01 M versene, a much greater lowering of concentration is required to obtain the same decrease of optical density as in experiments without versene.

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[1] A further examination of the protein release under various conditions will be published in connection with other investigations on the structure of the medullary granules. — In this experiment, the release of catechols was less at lowering of the sucrose concentration from 0.8 M to 0.5 and 0.3 M respectively than in the previously performed experiments, which might possibly be explained from the fact that in the preceeding experiments the granule suspensions had been kept for several hours at + 3.° C. before being used.

The release of proteins from granules does not only occur under the conditions mentioned above but on the whole at all the procedures which, in section I, have been shown to give a release of catechols. But any quantitative determinations of its magnitude have not yet been performed. In fact the optical density of the suspensions diminishes at this different treatments and this decrease becomes greater the greater the release of catechols is as in the experiments above.

Discussion

The experiments described in sections I and II show that adrenaline and noradrenaline are liberated from the medullary granules at lowering of solute concentration of the suspension medium and that the degree of this release is dependent on the concentration difference. It is near at hand to assume the observed catechol liberation to be an osmotic phenomenon but there is no direct evidence for this assumption. However, the fact that this liberation occurs in as well non-electrolytes as in electrolytes gives a support to this view. Furthermore, the time course of the release is such as could be expected from an osmotic effect on the granules, *viz.* per cent release rapidly reaches a certain level and then no further liberation takes place, just as the course of the percentage haemolysis curve under the same conditions for red blood cells. If the medullary granules behave as osmotic systems, it is reasonable to assume that they have different osmotic resistance. This is in agreement with the fact that rate of release curves (Figs. 2 and 3) were found to be S-shaped.

As shown in section III, the light scattering power of the medullary granules is pronouncedly diminished at lowering of solute concentration of the suspension medium. The changes in the granules leading to diminished light scatter take place very rapidly (within 2.5 minutes), are irreversible and coincide with the structural changes giving catechol liberation. These observations strongly support the assumption that the granules undergo lysis caused by decreased tonicity of the suspension medium, the degree of optical changes and of catechol release being an

expression for a lysis of all or of a definite fraction of the granules initially present. Furthermore, the fact that both these processes rapidly reach a certain level after which no further changes are found is difficult to explain without taking this assumption as basis of interpretation.

The experiments in section III further show that proteins leave the granules at lowering of tonicity of the suspension medium. When transferred to water the granules liberate approx. one half of their proteins, probably mainly in soluble form. As is evident from Fig. 14 there is good agreement between catechol release and protein liberation at lowering of sucrose concentration from 0.8 to 0.5 and 0.3 M respectively if the liberated proteins are expressed in per cent of the »watersoluble» proteins. This lends further support to the assumption that the medullary granules undergo lysis according to their osmotic resistance; the total amounts of catechols and »soluble» proteins in each granule undergoing lysis are released in consequence of the lytic changes.

If the above-mentioned interpretations of the experimental results are valid, the medullary granules would have a membrane impermeable to catechols and proteins within the granules. Is that the case, it could be expected that agents, well-known for high activity in disorganizing biological membranes, would give a release of catechols and of proteins from the granules owing to membrane destruction. This was verified by observations on the effect of detergents, digitonin, lecithinase A and of lysolecithin.

Detergents have pronounced injurious influences on cell membranes the destruction of which probably is caused partly by direct action on the oriented lipid layers and partly, or maybe even mainly, by their high protein-denaturing potency (Anson, 1939, Baker, Harrison and Miller, 1941, Hotchkiss, 1946, Ponder, 1946). The denaturing effect of detergents on proteins was shown by Anson to take place at very low detergent concentration (10^{-2} to 10^{-3} M) and yet to depend on the protein/detergent ratio, proteins being denatured to higher extents at lower ratios. In a similar way as well anionic as cationic detergents have a strong effect on the medullary granules giving catechol release

at 10^{-2} to 10^{-3} M concentrations; the degree of this release was found not to depend on the absolute amount of detergent but on the granule/detergent weight ratio, smaller amounts of granules requiring less detergent for a complete catechol liberation.

The effect of digitonin on the medullary granules showed the same characteristics as the detergent effect. This substance gives cytolysis probably on account of its complex formation with cholesterol in the cell membrane (cf. ADAM, 1941, SCHMIDT-THOMÉ, 1942, RUHENSTROTH-BAUER, 1951) but other causes cannot be excluded (cf. SMITH and PICKLES, 1941). The granules contain about 0.4—0.5 mg. free cholesterol per 100 mg. granules (wet weight; HILLARP and NILSON, 1954); however, its localization within the granules is not known.

A more specific action on the medullary granules is obtained by lecithinase A. This enzyme readily attacks the granules giving catechol release at very low concentrations (2 μg. bee venom lecithinase liberated more than 50 per cent of the total catechols in 40 mg. granules during 30 min.). Lecithinase A selectively removes an unsaturated fatty acid from lecithin and is also active on cephalin but no other lipids seem to be affected (FAIRBAIRN, 1945); its specificity and destructive potency is clearly shown in experiments with monolayers of lecithin (HUGHES, 1935). — The effect of this enzyme and of digitonin obviously indicates that phospholipids and free cholesterol are essential for the structural integrity of the granules. DATTA and MACFARLANE (unpublished, cited from MACFARLANE and SPENCER, 1953) have presented evidence that the permeability of mitochondria is connected with the presence of lecithin.

Treatment of the medullary granules with detergents, digitonin and lecithinase A does not only give catechol release but also protein liberation and changes in their optical properties, indicating lysis. These facts support the membrane-hypothesis presented above.

The causes of catechol release at lowering of pH of suspension medium below 5.5—6, at increasing the temperature and at freezing and thawing of the granules are more difficult to interpret. Damages to a membrane may naturally be the cause, but there is no evidence for this view. However, these injurious

influences also give liberation of proteins and diminished light scattering power of the granules. Thus, the catechol release at treatment of the medullary granules with agents of quite different nature in every examined case is accompanied by two other phenomena, protein liberation and reduced light scattering power. It seems difficult to explain this fact and the other data obtained from the experiments in any other way than on the basis of the following hyptothesis for which evidence has been brought forth above:

The medullary granules have a membrane with semipermeable properties. The membrane is impermeable to adrenaline and to noradrenaline in their stored form and inside this surface structure there is also a soluble protein fraction constituting a large part of the proteins in the granules.

It is not known in what form the catechols are stored in the granules. The existence of very high concentrations in these structures (about 0.25 M, or more than 40 mg./ml.; HILLARP and NILSON, 1954), the rapid release of the stored catechols for instance at transferring the granules to water and the high solubility in water suggest, however, that adrenaline and noradrenaline exist in soluble form within the granules or as a disassociable complex easily split under the different conditions giving catechol release. If the granules isolated in sucrose solution are transferred to water it is easy to obtain catechol concentrations in this medium amounting to 2000—3000 μg. per ml. On account of the low solubility of adrenaline in water (about 300 μg./ml.) it is hardly possible that the liberated catechols exist in the form of free bases; is is more likely that they exist as salts of an organic acid, as suggested previously (HILLARP and NILSON, 1954). However, there is no proof for this assumption.

As the catechol containing granules probably are of a mitochondrial nature (HILLARP, HÖKFELT and NILSON, 1954), the assumption of the existence of a semipermeable granule membrane is in good agreement with the results of investigations dealing with the mitochondrial membrane. Electron microscopical examinations have shown the mitochondria to be covered by a definite envelope (DALTON, KAHLER, KELLY and STRIEBICH, 1949, MÜHLETHALER, MÜLLER and ZOLLINGER, 1950, PALADE, 1952, 1953,

GLIMSTEDT and LAGERSTEDT, 1953, SJÖSTRAND, 1953); these morphological investigations, however, do not give any clues concerning the properties of the membrane. Several investigations dealing with this problem have presented strong evidence that the mitochondrial membrane are semipermeable and the permeability to different ions has been measured (CLELAND, 1952).

BERTHET, BERTHET, APPELMANS and DE DUVE (1952) have shown that liver mitochondria are impermeable to glycerophosphate and that they behave as osmotic systems undergoing lysis under influence of various physical and chemical agents. A release of proteins at sonic disintegration and at swelling of mitochondria in water has been demonstrated by HOGEBOOM and SCHNEIDER (1950, 1951) and by SCHNEIDER (1953) and this finding was interpreted as indicating the presence of a semipermeable membrane. Further evidence was attained by studies on the ability of mitochondria to maintain a concentration of sodium and potassium ions above that of the surrounding medium (MACFARLANE and SPENCER, 1953) and by studies on the effect of tonicity of the suspension medium on sarcosomes (SLATER and CLELAND, 1953).

The observations made by CLELAND (1952) show that there must exist a definite difference between sarcosomes and medullary granules concerning permeability to disodium hydrogen phosphate. The sarcosome membrane was found to be rapidly penetrated by this substance, but in the present work there was no evidence for such a penetration into the medullary granules in Sørensen buffer. Another finding by CLELAND, *viz.* that permeability to potassium chloride was drastically increased at lowering of pH below 6.5, may give a basis for an explanation of the observed catechol release at pH below 6.

Summary

The effects of various physical and chemical agents on the catechol containing granules isolated from cow adrenal medulla has been examined.

Adrenaline and noradrenaline are liberated from the granules under following conditions:

a) At lowering of the tonicity of the suspension medium, the degree of the release being dependent on the concentration difference.

b) At pH below 6.

c) At increased temperature and at freezing and thawing.

d) At treatment with as well anionic as cationic detergents in 10^{-2} to 10^{-3} M concentrations, the release being dependent on granule/detergent weight ratio.

e) At treatment with digitonin in low concentrations (10^{-3} to 10^{-4} M).

f) Under influence of small amounts of lecithinase A and lysolecithin.

The catechol release at treatment of the medullary granules with these agents of quite different nature is in every examined case accompanied by two other phenomena, protein liberation and reduced light scattering power of the granules.

On the basis of the data obtained it is assumed that the medullary granules behave as osmotic systems and that they undergo lysis according to different osmotic resistance. Evidence is further brought forth for the following conception of granule structure:

The medullary granules have a membrane with semipermeable properties. The membrane is impermeable to adrenaline and to noradrenaline in their stored form and inside this surface structure there is also a soluble protein fraction constituting a large part of the proteins in the granules.

The existence of very high catechol concentrations in the granules, the rapid release of the stored catechols for instance at transferring the granules to water and the high solubility of the liberated adrenaline and noradrenaline in water suggest that the sympathomimetic amines exist in soluble form within the granules or as a disassociable complex easily split under the different conditions giving catechol release. Furthermore, it is suggested that they may exist as salts of an organic acid, but as yet there is no proof for this assumption.

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